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Invited review

Nitric oxide: a newly discovered function on wound healing

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Abstract

Wound healing impairment represents a particularly challenging clinical problem to which no efficacious treatment regimens currently exist. The factors ensuring appropriate intercellular communication during wound repair are not completely understood. Although protein-type mediators are well-established players in this process, emerging evidence from both animal and human studies indicates that nitric oxide (NO) plays a key role in wound repair. The beneficial effects of NO on wound repair may be attributed to its functional influences on angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling. Recent findings from *in vitro* and *in vivo* studies of NO on wound repair are summarized in this review. The unveiled novel mechanisms support the use of NO-containing agents and/or NO synthase gene therapy as new therapeutic regimens for impaired wound healing.

Introduction

Wound repair is a well orchestrated and highly coordinated process that includes a series of overlapping phases: inflammation, cell proliferation, matrix deposition, and tissue remodeling. This involves a complex, dynamic series of events including clotting, inflammation, granulation tissue formation, epithelialization, neovascularization, collagen synthesis, and wound contraction[1]. Loss of a functional healing process could lead to severe disabilities. Accordingly, chronic, non-healing wound conditions represent a situation of major clinical importance. The series of pathological changes associated with several diseases ultimately leads to severely disturbed wound healing conditions[2]. Among those, the most prominent chronic wound impairments include decubitus or pressure ulcers, venous ulcers, and diabetic ulcers. The advent of molecular and cellular biology and the use of different modeling systems, most notably genetically engineered animals, have greatly extended our knowledge of wound repair. Inflammation, re-epithelialization, and granulation tissue formation are driven in part by a complex mixture of growth factors and cytokines, which are released coordinately into the wounds[1,2]. Besides these protein-type factors and mitogens, evidence is emerging for the important role of small diffusible molecules such as nitric oxide (NO) in wound repair[3]. In this review, we summarize the current knowledge of the modulating functions of NO on wound repair.

Chemistry and biosynthesis of nitric oxide

NO is a highly diffusible intercellular signaling molecule implicated in a wide range of biological effects. It is generated by the enzyme nitric oxide synthase (NOS), which catalyzes the conversion of *L*-arginine to *L*-citrulline[4]. Three NOS isoforms have been characterized, each encoded by different chromosomes. Two enzyme isoforms are constitutively expressed (endothelial and neuronal NOS), whereas one isoform is an inducible enzyme (iNOS), initially found in macrophages. All three NOS isoforms exist in their active form of homodimers of two domains: a C-terminal reductase domain, and an N-terminal oxygenase domain with molecular masses of approximately 135 kDa (eNOS), 150–160 kDa (nNOS), and 130 kDa (iNOS)[4]. The reductase domain contains binding sites for one molecule each of nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), and flavin dinucleotide (FAD), in close homology with cytochrome P-450 reductase, whereas the oxygen domain binds heme, the essential cofactor tetrahydrobiopterin (BH4), and the substrate *L*-arginine[4].

Between these two regions lies the calmodulin (CaM) binding site, which plays a key role in both the structure and function of the enzyme. The constitutive isoforms (eNOS and nNOS) are permanently active, generating low concentrations of NO (in nmol/L range). Their enzymatic activities are regulated by intracellular calcium fluxes or exogenous calmodulin. The expression, transcription, and function of the iNOS is induced by a variety of cytokines, growth factors, and inflammatory stimuli on target cells which lead to the release of much higher levels of NO (in μmol/L range), which is involved in host immune response.

All three NOS isoforms are expressed in skin tissue[3]. Expression of nNOS has been observed in keratinocytes and melanocytes; eNOS can be detected in keratinocytes of the basal epidermal layer, dermal fibroblasts, endothelial capillaries, and eccrine glands; and iNOS can be induced in keratinocytes, fibroblasts, Langerhans, and endothelial cells. Accordingly, NO participates in the regulation of skin homeostatic functions such as circulation, sunburn erythema, and maintenance of the protective barrier against microorganisms.

Nitric oxide and wound healing

L-Arginine, the substrate for NOS, was first noted to enhance wound healing in 1978[5]. Subsequently, dietary *L*-arginine intake has been shown to improve collagen deposition and wound strength in both animals and humans[6–8]. This effect of *L*-arginine may be due in part to its conversion to *L*-ornithine through the action of arginase, an enzyme that may compete with NOS for *L*-arginine and thereby help regulate NO production during wound healing[9]. However, the finding that *L*-arginine intake does not improve collagen deposition in iNOS-deficient mice to the same extent as in wild-type littermates implicates that part of *L*-arginine's effect involves NO directly[10].

Accumulating evidence indicates that NO plays a key role in normal wound repair (Figure 1)[11–18]. Production of nitrite (NO_2) and nitrate (NO_3), the stable NO metabolites, are elevated early in the fluid of subcutaneous wounds[11], and urinary nitrate excretion increases in a sustained manner after excisional wounding[12]. Furthermore, the presence of nitrite and nitrate is directly correlated with collagen deposition within the wound and in dermal fibroblasts, suggesting that NO synthesis is critical for wound collagen accumulation and acquisition of mechanical strength[11,13,14]. All three NOS isozymes are involved in the wound healing process. Both iNOS and nNOS mRNA and protein expression are increased in cutaneous wounds[15,16]. Our recent findings demonstrate that there is a significant increase of cutaneous eNOS protein expression as well as constitutive NOS enzymatic activity after excisional wounding in normal mice[17]. Consequently, an NO deficiency directly contributes to wound healing impairment. Inhibition of NOS by competitive inhibitors, either applied to the wound surface[18] or given systemically[11], decreases collagen deposition and breaking strength of incisional wounds and impairs the healing.

Consistent with these findings, studies with targeted disruption of NOS genes have revealed that the excisional wound closure is delayed by 30% in both eNOS and iNOS knockout mice compared to their wild-type littermates[19,20]. Conversely, adenoviral vector-mediated gene transfer of iNOS to the wound site of iNOS knockout mice completely reversed the delayed healing[20].

Finally, there are strong correlations between reduced cutaneous NO levels and impaired wound healing under disease conditions such as diabetes[11,14,17,21], malnutrition[13], and chronic steroid treatment[18]. Diabetic wound healing impairment is one of the most well-known chronic wound situations. Studies of ours and others demonstrate that cutaneous eNOS expression, constitutive NOS activity, and/or NO levels are significantly decreased in streptozotocin (STZ)-induced type 1 diabetic animals[11,14,17,21]. In fact, our findings indicate that the augmented cutaneous eNOS protein expression and constitutive NOS activity observed in normal animals in the healing process are absent in the type 1 diabetic mice[17]. These findings suggest that impairment of wound-induced endogenous NOS expression and NOS activity is responsible for reduced cutaneous NO bioavailability in type 1 diabetic animals (Figure 1). In agreement with the above notion, cutaneous gene therapy of eNOS or manganese superoxide dismutase (MnSOD) restored eNOS protein and NO levels and accelerated the wound healing rate in STZ-induced diabetic mice[17]. Similarly, the NO donor molsidomine (*N*-ethoxycarbomyl-3-morpholinyl-sidnonimine) or NO releasing poly (vinyl alcohol) hydrogel dressings are also shown to partially restore such healing impairment in STZ-induced diabetic rats[22,23]. Collectively, impairment of skin NO function represents an important factor for delayed wound healing in diabetes and strategies aimed at restoring cutaneous NO bioavailability with NO donors or NOS gene therapy may serve as effective means for diabetic wound healing.

Mechanisms of nitric oxide on wound healing

NO and angiogenesis Angiogenesis, the process of forming new microvessels, is an important component of normal

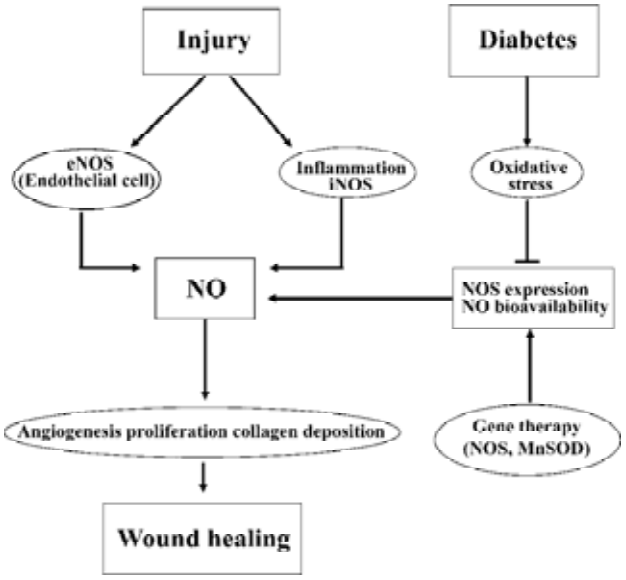

Figure 1. Nitric oxide (NO) as a signaling molecule accelerates wound healing. Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; VEGF, vascular endothelium growth factor.

wound repair. NO plays a central role in this process[24] as it increases angiogenesis in ischemic murine tissues[25]. Conversely, NOS inhibitors impair angiogenesis in granulation tissue during gastric ulcer healing[26] and suppress capillary organization *in vitro*[27].

NO is also vital to the activity of pro-angiogenic cytokines. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor which involves the modulation of NO generation[28]. VEGF increases NO production via upregulation of eNOS[29,30]. Conversely, the angiogenic effect of VEGF also depends on NO as pharmacological blockade of NOS prevent both VEGF-induced endothelial cell proliferation and mitogen-activated protein (MAP) kinase[31]. VEGF-stimulated endothelial cell migration, decreased adhesion, and organization are also dependent on NO[32,33]. Keratinocytes are the major source of VEGF expression upon cytokine stimulation[34], which is blocked by iNOS inhibitors both *in vitro* and *in vivo*[35]. NO has also been shown to downregulate protein kinase C (PKC)-induced VEGF expression in smooth muscle cells by interfering with the binding of AP-1[36] and to participate in the conversion of VEGF from an inert to an angiogenic form[37]. Interestingly, NO is also involved in VEGF-independent angiogenesis mechanisms. Evidence includes the role of NO in monocyte-induced angiogenesis induced by monocytes[38], substance P[39], and transforming growth factor (TGF)-β1[40]. Taken together, these studies suggest a vital role of NO in post-wound angiogenesis.

NO and inflammation NO has been shown to modulate chemoattractant cytokines that initiate post-wound inflammation, including interleukin (IL)-8[41], TGF-β1[42], monocytes, and neutrophils[43] that contribute to wound chemoattraction. Once monocytes and neutrophils are attracted to the site of a wound, they are activated and begin to produce TNF-α and IL-1, both of which are implicated in wound healing[33]. Because IL-1 is a potent chemoattractant for keratinocytes, the modulation of IL-1 by NO may usher keratinocyte recruitment, proliferation, and differentiation. Taken together, NO modulation of inflammation-associated cytokines may affect the inflammatory phase of wound healing.

NO and cell proliferation, differentiation, and apoptosis NO affects proliferation, differentiation, and apoptosis in a number of cell types involved in wound healing. The iNOS inhibitor N^{ω}-imino ethyl *L*-lysine (*L*-NIL) has been found to decrease proliferation in keratinocytes at the wound edge[44]. Indeed, treatment of murine wounds with *L*-NIL leads to delayed re-epithelialization with atrophied hyperproliferative epithelium seen at the wound edge[45]. Conversely, the NO donor sodium nitroprusside (SNP) significantly increases fetal bovine serum-induced thymidine incorporation into the DNA of human dermal fibroblasts and enhances fibroblast growth factor- or platelet-drived growth factor-induced DNA synthesis[46]. Furthermore, low levels of NO increase keratinocyte proliferation *in vitro*[44], an effect that is mimicked by 8-bromo-cGMP[33], an analog of NO second messenger cGMP. NO also modulates keratinocyte apoptosis induced by irradiation of keratinocytes with ultraviolet B light as addition of NOS inhibitors to irradiated keratinocytes increases apoptosis, an effect that is reversed by the NO donor *S*-nitroso-penicillamine[47]. It appears that both inducible and constitutive NOS are involved in this process. Furthermore, NO has been shown to stimulate the proliferation of endothelial cells, protect endothelial cells from apoptosis, and mediate VEGF production[34]. These effects of NO on endothelial cells may also be related to another facet of wound healing, namely angiogenesis. In contrast, NO may also affect fibroblast proliferation. For instance, NO donor SNAP has been reported to decrease the proliferation of normal dermal fibroblasts in rats[48] while increase their proliferation in mice[49], even though the reasons for such discrepancy are not clear. Altogether, the above studies suggest that NO affect the proliferative phase of wound healing.

NO and matrix deposition and remodeling The final phases of healing require increased collagen synthesis and

deposition, and a link between NO and collagen deposition has been described[11]. In most studies, treatment with NO donors, dietary *L*-arginine, or iNOS overexpression via gene therapy increased the collagen content of experimental wounds[10,11,50,51]. Indeed, treatment with a NO donor has been shown to increase collagen formation in fibroblasts derived from both normal and wound skin, which was decreased following NOS inhibition[51]. The effect of NO may primarily be due to the posttranslational enhancement of collagen synthesis rather than *de novo* transcription of the relevant collagen genes[51].

Mechanisms of wound nitric oxide dysfunction

Although impaired NO function contributes to delayed wound healing in diabetes, the mechanisms of cutaneous NO dysfunction in this setting is unclear. In diabetes, causative factors for hyperglycemia-induced organ damage include the activation of the polyol pathway, nonenzymatic glycation, activation of PKC pathway, and increased hexosamine pathway flux[52]. However, previously there was no apparent common element linking these mechanisms. Recent studies suggest that these different mechanisms may be linked by a single cellular process: an overproduction of superoxide induced by sustained hyperglycemia[53]. Sustained hyperglycemia is known to increase vascular superoxide levels, resulting in cardiovascular dysfunction[54]. Superoxide produced in the vasculature rapidly inactivates NO and thus reduces its bioavailability in diabetic vasculature[55]. Independent strategies aimed at reducing superoxide levels have been shown to prevent high glucose-induced PKC activation, formation of advanced glycation end-products (AGEs), sorbitol accumulation, and NFκB activation, resulting in improvement of endothelium-dependent NO-mediated vasodilation[53,54]. These results indicate that increased superoxide levels are a key factor in vascular NO dysfunction in diabetes. However, whether sustained hyperglycemia increases cutaneous O_2^- levels and the mechanisms by which cutaneous NO levels are decreased in diabetes is unknown. Our recent studies demonstrate that glucose concentration-dependently increases superoxide levels in normal mouse skin and there is a marked increase of cutaneous superoxide levels in streptozotocin-induced type 1 diabetic mice[17]. Furthermore, cutaneous gene therapy of MnSOD significantly reduced superoxide and increased NO levels, resulting in accelerated wound healing in this model. These results provide the direct evidence that increased cutaneous superoxide contributes to reduced NO bioavailability and wound healing impairment in diabetes (Figure 2).

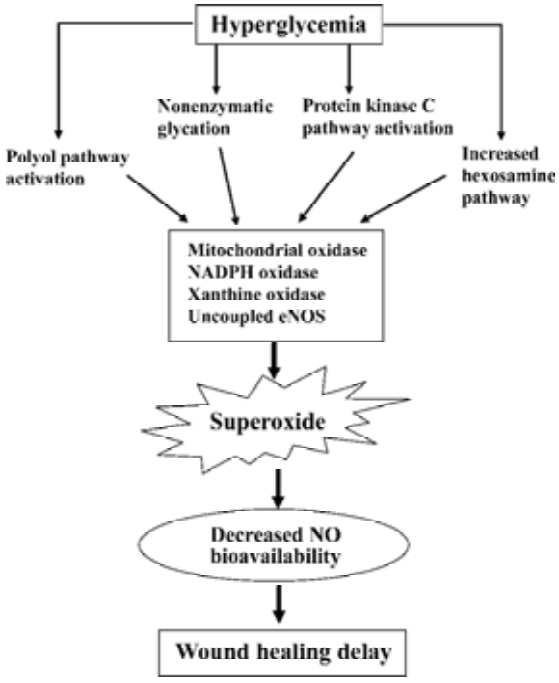

Figure 2. The proposed mechanism by which superoxide contributes to decreased NO bioavailability and wound healing impairment in diabetes.

Future directions

Although a central role for several protein-type growth factors and mitogens on wound repair has been well-established for many years, the application of these factors in the treatment of wound healing has not provided a breakthrough in the clinical arena[1,2]. One possible reason for the failure of markedly accelerating closure of chronic wounds may be due to increased protease activities in the wound fluids, which may impair the ability of endogenous and exogenously applied growth factor proteins to stimulate healing. In contrast, NO may represent a novel target molecule to circumvent these difficulties. Because NO is a short-lived gas molecule, maintaining an effective level of NO at the wound site is an obvious problem for clinical therapy. In recent studies we have demonstrated that gene therapy of NOS or SOD is effective in restoring cutaneous NO levels and accelerating wound healing in diabetic mice[17]. Gene therapy strategies aimed at increasing NO or reducing superoxide levels may represent an effective means of reversing cutaneous NO deficiency at the wound site for healing refractory wounds in diabetes and other diseases. Future preclinical studies are warranted to optimize the designs and regimens before clinical trails can be conducted and the ultimate translation of basic science to the clinical settings for human gene therapy.

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