Role of nitric oxide in wound repair

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Abstract

After injury, wound healing is essential for recovery of the integrity of the body. It is a complex, sequential cascade of events. Nitric oxide (NO) is a small radical, formed from the amino acid L-arginine by three distinct isoforms of nitric oxide synthase. The inducible isoform (iNOS) is synthesized in the early phase of wound healing by inflammatory cells, mainly macrophages. However many cells participate in NO synthesis during the proliferative phase after wounding. NO released through iNOS regulates collagen formation, cell proliferation and wound contraction in distinct ways in animal models of wound healing. Although iNOS gene deletion delays, and arginine and NO administration improve healing, the exact mechanisms of action of NO on wound healing parameters are still unknown. The current review summarizes what is known about the role of NO in wound healing and points out path for further research. © 2002 Excerpta Medica, Inc. All rights reserved.

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Nitric oxide (NO) is a short-lived free radical that is involved in many important biological functions. As a testimony to the rapidly expanding knowledge about its multiple biological roles, NO, after its discovery in 1987, was named molecule of the year in 1992 [1].

NO is formed from the terminal guanidino nitrogen atom of arginine. The guanidino nitrogen accepts five electrons in an oxidation process requiring molecular oxygen, resulting in formation of NO and citrulline. L-hydroxy-arginine is formed as an intermediate product (Fig. 1). Nitric oxide synthases (EC 1.14.13.39) the enzymes responsible for this conversion, are homodimeric flavoprotein enzymes (130 – 150 kDA subunits). They require tetrahydrobiopterin, flavine mononucleotide (FMN), flavine adenine dinucleotide (FAD), nicotinamide-adenine-dinucleotide phosphate (NADPH) and oxygen as co-factors for full activity [2]. The NO synthases exist in three distinct isoforms, two constitutives (endothelial and neuronal) and one inducible isoform. The constitutive isoforms are permanently active generating low concentrations of NO. Their enzymatic activities are regulated by intracellular calcium fluxes or exogenous calmodulin [Fig. 2(a)]. The expression, transcription, and function of the inducible isoform (iNOS) is induced by a variety of cytokines, growth factors and inflammatory stimuli on target cells which leads to release of high levels of NO compared to the amounts generated by the constitutive isoforms [Fig. 2(b)]. Therefore, the regulation of iNOS takes places mainly at the gene level. The constitutives and inducible isoforms have only about 50% sequence homology [2].

The high amounts of NO formed by the inducible isoform account for some of its detrimental effects in inflammatory situations such as sepsis [3]. iNOS is also released during wound healing, burn injury, endotoxin exposure, arthritis and inflammatory bowel diseases.

NO expression in wounds

L-arginine—the substrate

The metabolism of NO is critically dependent on the metabolism of L-arginine since this amino acid is the sole substrate for NO synthesis. Although some reports have described spontaneous, non-enzymatic dependent NO formation [4], this is probably not relevant to wound healing. Levels of arginine, a semiessential amino acid [5], become critically low after wounding [6,7].
It is important to note that L-arginine can also be metabolized in wounds via arginase, which is present in high concentrations in wound fluid due to its release by wound macrophages [8] (Fig.1). Through the action of arginase, ornithine is formed which is a precursor for proline and polyamine generation [8]. It had been hypothesized that this may be a significant pathway for wound healing since proline serves as substrate for collagen synthesis, whereas polyamines are involved in cell proliferation [9,10].

Arginine utilization by means of arginase may also down regulate NO synthesis during wound healing by substrate depletion [11]. In vitro work has indeed shown that arginine utilization and breakdown by arginase can impair NO formation in macrophages because of substrate limitation [12]. To date, however, no in vivo data are available proving that this alternative pathway of L-arginine catabolism affects wound healing.

There are also strong regulatory mechanisms between the different arginine metabolizing pathways. L-hydroxyarginine and nitrite, the intermediate and end product respectively of the NO pathway, are both strong arginase inhibitors [13,14]. Further, urea, end product of arginase activity, inhibits NO formation [15]. Distinct cytokines favor the various degradative pathways. TGF-β and IL-4 increase arginase and inhibit iNOS activity, whereas IFN-γ, IL-1, LPS work inversely [16–18].

As NO is difficult to measure directly, stable metabolites are used as surrogates for NO formation. Nitrite and nitrate, two widely used stable end products, can be measured in wound fluid [19]. However, these measurements should not be translated as equimolar formation of NO since nonenzymatic NO formation can occur [20]. Several other direct or indirect detection methods can be performed such as immunohistochemistry, direct measurement of enzyme content and/or activity, peroxynitrite formation in tissue, gene expression and others. However no study has so far investigated arginine kinetics after wounding taking into account local and systemic arginine metabolism [21,22].
NO—time course

Before NO was known, Albina et al. investigated arginine metabolism in wounds and demonstrated increased citrulline formation which was imputed to an arginine deiminase-like activity [23]. Up to that time, this pathway of arginine disposal had been described only in bacteria and fungi. Subsequently, generation of NO during wound healing was deduced by demonstrating increased urinary nitrate secretion after wounding [24]. Thereafter several studies confirmed these data and extended it to healing after burn injury [25,26]. In these models, urinary nitrate levels remained elevated until complete healing had occurred. Later experiments confirmed that the highest NOS activity occurs during the early phases of wound healing [8].

By using a polyvinyl alcohol sponge model in rats, a progressive accumulation of nitrate/nitrite in wound fluid can be shown, suggesting sustained NO synthesis [27]. However species-specific differences in NO formation exist [28].

With the development of NOS isoform specific antibodies and primers for transcriptional and translational analysis it was demonstrated that iNOS expression is highest in the early phase after acute inflammation. RT-PCR and Northern Blotting detect iNOS during the first 5 days in rat models of healing [29,30].

It is conceivable that the majority of NO synthesis is due to the inflammatory cells present during the early phase of healing, especially macrophages [31]. However, fibroblasts, keratinocytes and endothelial cells contribute to ongoing NO synthesis but to a lesser degree [32,33]. Therefore the overall time course of iNOS activity and NO generation during wound healing has to be viewed as a decreasing curve over time (Fig. 3).

NO—the regulation

Although the in vitro signals of iNOS induction are well elucidated, little is known of the in vivo signals during wound healing. Of the numerous cytokines and growth factors secreted and released into the wound environment, interleukin-1, TNF-α, and γ-interferon are the most likely inducers of iNOS. Wound fluid, as a biological reflection of the wound environment, induces NO synthesis in a variety of cells [34].

Although iNOS expression is high during the early phases of wound healing, little is known about the down-regulation of iNOS activity at the wound site during the later phases of healing. Presumably iNOS activity can be down regulated by the resolution of the inflammatory response or by cytokine signaling. It is likely that colonized or infected wounds with continued inflammatory responses would continue to generate large amounts of NO, although this has not been studied directly. TGF-β1 is one of the strongest iNOS inhibitors during wound healing [35]. However even during the inflammatory phase of wound healing there is counter regulation of NO synthesis, as demonstrated by the presence of an unknown factor which reduces iNOS activity, but not by substrate depletion [31].
Mechanism of action

NO acts by way of multiple and different mechanisms. Some of its effects are due to its chemical reaction with oxygen leading to formation of reactive radical species [36]. Others are due to its affinity with heme or metal containing enzymes such as the Fe in guanyl-cyclase [Fig. 2(a)]. As a review of the complex NO chemistry is beyond the scope of this article, we will concentrate on biological aspects relevant to wound healing.

NO has been shown to be cytostatic to multiple cell types including endothelial cells, smooth muscle cells, hepatocytes, and fibroblasts [37–39]. Depending on the cell type this effect can be cGMP dependent [40] or independent [41]. Target enzymes include complexes I and II of the respiratory chain [42] and ribonucleotide reductase [43], a rate-limiting enzyme in the DNA synthetic pathway. NO is cytostatic in large doses. However, several studies have shown that NO can stimulate cell proliferation when added in low concentrations [44,45]. Recent evidence suggests that NO can also reduce cell proliferation by inhibiting ornithine-decarboxylase activity, the rate limiting enzyme for polyamine formation [46].

NO also regulates gene expression [47,48] and cellular differentiation [49,50]. Regulation of gene expression by NO probably occurs indirectly, through amplification of other regulatory mechanisms [51]. For example, although NO is critical for wound collagen deposition, clear-cut enhancement of collagen synthesis or gene expression has not been found (see the following). Collagen metabolism and accumulation are tightly regulated through the activity of collagenases and their inhibitors, tissue inhibitors of metalloproteinases (TIMP). Inhibiting the collagenolytic pathway can enhance collagen accumulation. Addition of the NO donor SNAP to rat mesangial cells increases gelatinase A activity [52] whereas rat fibroblasts collagenase activity is unaffected by SNAP [41]. Another potential mechanism of posttranslational collagen regulation by NO is regulation of protein kinase C (PKC) activity [53,54]. By inhibiting PKC activity, NO could down-regulate PKC-related collagen synthesis in fibroblasts.

Function of NO in Wound Healing

There is increasing evidence for a functional role of NO in wound healing. Inhibition of iNOS by competitive inhibitors decreases collagen deposition and breaking strength of incisional wounds and impairs the healing of other wound models [55–57]. When rats are fed an arginine-free diet, wound healing is impaired: conversely, when humans and animals are fed an arginine-enriched diet there is improved healing as measured by collagen deposition and breaking strength [58–60]. Arginine-supplemented rats have higher levels of NO metabolites in their wound fluid, strongly suggesting that the supplemental arginine is metabolized, at least partly, by way of NO synthesis [61]. Finally, use of NO donors also improves incisional and excisional wound healing in rats [62–64]. The data demonstrating that NO has a positive regulatory effect on repair is summarized in Table 1.

The mechanism of action of NO on wound healing remains unclear. However, there is also data suggesting that at least some effects of NO on wound healing might be systemically mediated: (1) Arginine-free nutrition inhibits LPS-induced NO synthesis in several organs not only at the wound site [65]; (2) NO mediates inflammation-induced edema formation and inhibits cell infiltration into granulomas [66,67]; (3) the effect of NO on wound healing is not only iNOS-mediated since eNOS knock-out mice also demonstrate impaired healing [68]; and (4) iNOS inhibitors have a high lethality in high concentrations [55].

In vitro studies of fibroblasts derived from keloids and hypertrophic scars demonstrate low constitutive NOS expression, thus stimulating higher cell proliferation which is responsible for the high cellularity characteristic of these disorders. In vivo, keratinocyte proliferation is iNOS-dependent [29] and wound reepithelialisation is also NO dependent probably mediated indirectly by way of VEGF [69]. Interestingly, induction of iNOS in keratinocytes is paralleled by induction of GTP-cyclohydrolase I, the rate-limiting enzyme for tetrahydrobiopterin formation, which is essential for full iNOS activity [70].

Collagen synthesis correlates with NO synthesis during wound healing. iNOS inhibition impairs whereas NO ad-
ministration and iNOS transfection enhance matrix synthesis [56,62,71]. Further, wound derived fibroblasts are characterized by a distinct phenotype where endogenous iNOS expression correlates with increased collagen synthesis [32].

Wound contraction is a major contributor to closure of open wounds. In excisional wounds closure is delayed by iNOS inhibition [69]. In vitro studies showed that NO induces a locomotory phenotype in keratinocyte [72].

Wound healing is characterized by the organized secretion of growth factors. This represents a potential target for regulating wound healing. Little is known whether NO can directly affect growth factor or cytokine secretion, activation or time of action. Arginine is known to down-regulate TNF-α after trauma thereby affecting outcome [73]. TGF-β and EGF directly and indirectly down-regulate NO or the NO mediated effects [74].

Recently we have examined if lack of iNOS gene expression alters wound cytokine expression. Using nonisotopic in situ hybridization quantitative analysis we studied eNOS, basic FGF (bFGF), TGF-β1, TNF-α, VEGF, and IL-4 expression in incisional wounds and compared expression in wild type and iNOS-KO mice. eNOS and bFGF expression nearly doubled on POD 7 in iNOS-KO incisions and remained two- through threefold elevated thereafter. TGF-β1 expression was increased approximately 50%–100% in iNOS-KO wounds on POD 5 and 7. VEGF and IL-4 expression was elevated by 25%–100% in wild-type compared with iNOS-KO animals at all time points. We hypothesize that the over-expression of TGF-β1 and eNOS may represent mechanisms in iNOS-KO mouse to compensate for their loss of functional iNOS, resulting in incisional wound healing equivalent to controls. The impaired expression of VEGF and IL-4, on the other hand, may partially explain the delayed excisional wound healing noted in these animals (Most and Barbul, in press).

**Impaired wound models**

After the discovery that NO is synthesized during wound healing and that inhibition of its production impairs healing, the next step was to investigate whether there is a correlation between NO and outcome of healing. Several impaired wound models were used to seek such correlation.

In diabetes at least three studies have demonstrated decreased formation of NO metabolites in the wound environment [75–77]. It is not clear whether this decrease is due to the lesser inflammatory response characteristic of diabetes or to a net decrease in NO formation by all wound cells. L-arginine as well as NO donors can partially reverse the impaired healing of diabetes and in parallel restore wound NO levels toward more normal values [62, 77]. More work needs to be carried out to confirm whether these agents might serve as future treatment options.

Malnutrition and radiation induced injury are other conditions associated with impaired or delayed healing [78] (Schaffer, unpublished). Steroids, strong inhibitors of healing, alter arginine metabolism by impairing both the iNOS and the arginase pathways [79].

iNOS knock-out mice demonstrate delayed closure of excisional wounds which can be reversed by transfection with iNOS-cDNA [80]. Surprisingly however, there is no effect on collagen deposition or breaking strength in incisional wounds in iNOS knockout mice [81]. Supplemental L-arginine does not enhance wound healing in iNOS knock-out mice, suggesting that metabolism of arginine via iNOS is an essential pathway in the positive effects of arginine on healing [61]

eNOS knock-out mice also demonstrate delayed healing in excisional wound models [68]. Wound fluid extracted from these wounds, induces a lesser angiogenic response in the cornea angiogenesis models than controls, underscoring the importance of eNOS for neoangiogenesis during wound healing.

**Future directions**

Treatment of acute and chronic wound failure is still a major unresolved goal. 25% of the delays in hospital discharge can be attributed to wound failure. Wound dressings delivering NO have been used in experimental models [64]. Recently NO-releasing NSAID have been investigated in experimental wound healing [82]. Combining NO to a NSAID enhanced collagen synthesis that was otherwise decreased when NSAID was administered alone [83]. High dose arginine supplementation as a means of elevating wound NO synthesis and enhancing wound healing awaits wider application in the clinical arena.

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