PERSPECTIVE ARTICLE

Biological fate and clinical implications of arginine metabolism in tissue healing

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ABSTRACT

Since its discovery in 1987, many biological roles (including wound healing) have been identified for nitric oxide (NO). The gas is produced by NO synthase using the dibasic amino acid L-arginine as a substrate. It has been established that a lack of dietary L-arginine delays experimental wound healing. Arginine can also be metabolized to urea and ornithine by arginase-1, a pathway that generates L-proline, a substrate for collagen synthesis, and polyamines, which stimulate cellular proliferation. Herein, we review subjects of interest in arginine metabolism, with emphasis on the biochemistry of wound NO production, relative NO synthase isoform activity in healing wounds, cellular contributions to NO production, and NO effects and mechanisms of action in wound healing.

According to data from the United States National Center for Health Statistics, 40.3 million inpatient and 31.5 million outpatient surgical procedures were performed in the United States, in 1996. In addition to these procedures, annually in the United States there are an estimated 50 million acute traumatic wounds;[1,2] diabetic foot ulceration costs approximately $150 million;[3] and the total financial burden of wound care in the United States is estimated at more than $20–25 billion. Meanwhile, in the United Kingdom, wounds are estimated to cost the National Health Service approximately £1 billion per annum.[4] Annually, there are up to 24,000 admissions in the United Kingdom for patients suffering from diabetic foot ulceration, at a cost of approximately £17 million[5] and the problem of chronic venous ulceration costs the National Health Service approximately £400 million.[6]

Since its discovery in 1987, many biological roles (including wound healing) have been associated with nitric oxide (NO).[7] Clinical states where wound resolution is impaired have been the focus of NO research in the hope that modulation may improve healing. The diabetic state is characterized by reduced NO synthase (NOS) expression and depleted NO availability in wounds.[8–11] The biological role of NO in wound healing and epithelial restitution is reviewed here with an emphasis on diabetic healing and a focus on potential therapeutic interventions.

BIOCHEMISTRY OF NO

NO has many forms including gas, free radical, neurotransmitter, and intracellular signaling molecule.[12–14] It may be derived via one of two distinct but interactive processes: metabolism of NO-generating compounds or synthesis by NOS (using the dibasic amino acid L-arginine as a substrate). Arginine is provided through nutritional intake or de novo synthesis from other molecules (e.g., citrulline).[15] Thus, arginine is a conditionally essential amino acid in the context of wound healing.[16]

The site of action of all NOS is the terminal guanidine nitrogen on L-arginine, which is oxidized to produce NO and citrulline (Figure 1). All three isoforms of NOS are homodimers including a C-terminal reductase domain, an N-terminal oxygenase domain, and a central calmodulin-binding site that has a key role in regulation.[18–20] They are all homodimeric flavoprotein enzymes (130–150 kDA subunits) requiring flavine mononucleotide, flavine adenine dinucleotide, nicotinamide-adenine-dinucleotide phosphate, tetrahydrobiopterin, and oxygen as co-factors.[21] However, the isoforms display spatial segregation[22–27] as well as constitutional[21–28] and regulatory heterogeneity.[29]

The two constitutive isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), produce concentrations of NO in the nmol/L range in a tonic fashion.[30] The inducible

bFGF	Basic fibroblast growth factor
EGF	Epidermal growth factor
eNOS	Endothelial NOS
IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Inducible NOS
ko	Knockout
LPS	Lipopolysaccharide
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal NOS
STZ	Streptozotocin
TGF-β1	Transforming growth factor-β1
VEGF	Vascular endothelial growth factor

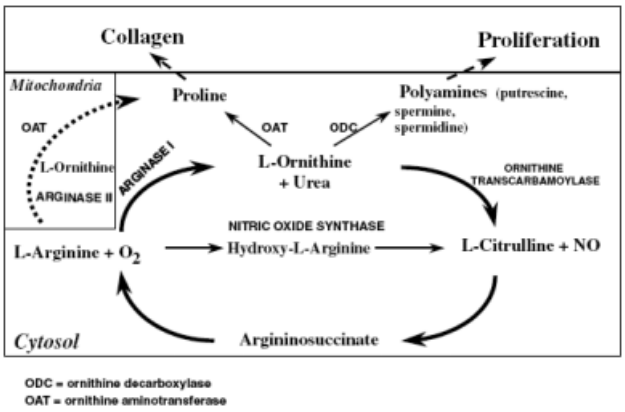

Figure 1. Schematic representation of the metabolism of arginine. With permission from Witte and Barbul.[17]

form (iNOS) is activated by cytokines, growth factors, inflammatory stimuli (e.g., microbial products), and hypoxia (Table 1).[26,27] Constitutive and inducible forms exhibit approximately 50% sequence homology.[21] While the constitutive enzymes require intracellular calcium and calmodulin regulation,[31] the inducible isoform is maintained active by calmodulin independent of local calcium concentrations.[32] Therefore, NO production by iNOS is limited only by enzyme, substrate, and co-factor availability and their mutual thermodynamic interaction. Detrimental effects of NO seen in severe inflammatory conditions are explained by high concentrations of iNOS-derived NO outside the influence of tight regulation.[32–34]

The first NOS to be discovered was nNOS. The inducible form is typically not expressed in cells in their basal state.[26] The inducible form was initially isolated from activated macrophages, and can be expressed in virtually all tissues under the appropriate conditions.[23,27]

ARGININE AND WOUND HEALING—AN ESSENTIAL AMINO ACID

Although the mechanism was unclear, Seifter et al.[35] noted that L-arginine was conditionally essential following tissue trauma in 1978. Since then, it has been established that a lack of dietary L-arginine delays experimental wound healing while resumption of oral intake improves collagen deposition and wound strength in both animal models and patients.[36–40] Furthermore, parenteral supplementation may increase collagen deposition and breaking strength in rodent and human dermal incisions.[36–40]

Arginine can be metabolized to urea and ornithine by arginase-1 (Figure 1). This pathway is important in the healing process as it generates proline, a substrate for collagen synthesis, and polyamines, that stimulate cellular proliferation.[41–43] Alternatively, L-arginine may be metabolized by NOS isoforms and the NO produced has arguably more important roles in tissue repair, e.g., supplemental L-arginine does not improve the impaired healing of iNOS knockout (ko) mice—showing that without iNOS activity, arginase activity alone is insufficient.[44] However, the dynamic competition for L-arginine between arginase and NOS in the wound milieu is such that depletion of the amino acid by arginase limits NOS activity.[45] Similarly, urea, an end product of the arginase pathway, inhibits NO formation.[46] Meanwhile, products of NO synthesis (e.g., L-hydroxy-arginine and nitrite) inhibit arginase.[47,48] Various cytokines exert control on the degradative pathways available to L-arginine. Transforming growth factor (TGF)-β and interleukin (IL)-4 both increase arginase function and inhibit iNOS activity while interferon-γ (IFN-γ), IL-1, and lipopolysaccharide (LPS) work inversely.[45,49,50]

Table 1. Table showing tissue distribution of NOS isoforms

	nNOS	eNOS	iNOS	References
Skin				
Keratinocytes	√	√	√	22–24, 26
Melanocytes	√			22–24
Fibroblasts		√	√	22, 26
Endothelial Cells		√	√	22, 26
Eccrine glandular Cells		√		22
Macrophages and neutrophils			√	23, 27, 33, 34
Nerve				
Neurons	√	√		22–25
Muscle				
Cardiac myocytes		√		25
Skeletal myocytes	√			22–24
Organs				
Pancreas	√			22–24
Kidney	√			22–24

EVIDENCE FOR WOUND NO PRODUCTION

Smith et al.[51] deduced the production of NO in wounds by demonstrating increased urinary nitrate production. Animal and patient studies confirmed these results in burn injury.[51–54] Production of nitrite and nitrate, the stable NO metabolites, is elevated early in subcutaneous wounds.[8] Levels of these metabolites (and overall NO production) correlate with collagen deposition as well as the synthetic and contractile properties of fibroblasts.[9] This suggests that NO synthesis is critical for reconstitution and the acquisition of mechanical strengths.[8,9,55]

Expression and activity of all three NOS isoforms are increased during the wound healing process.[10,24–26] Administration of NOS inhibitors such as *S*-methyl isothiouronium and aminoguanidine hemisulfate reduces local nitrite/nitrate concentrations, collagen deposition, and wound tensile strength.[8] After experimentally induced burn wounds in mice, epithelial proliferation, collagen formation, and quality of granulation tissue were all reduced as a result of NOS inhibition.[56] Even topical

administration of NOS inhibitors decreases collagen deposition and breaking strength of incisional wounds.[57]

Variable importance of iNOS activity in excised vs. incised wounds

It is known that optimal anastomotic healing requires the induction of iNOS gene expression in the intestine.[58] Furthermore, iNOS gene transfection via subcutaneously implanted sponges in rats is associated with enhanced cutaneous wound collagen accumulation.[59] Closure of excised wounds is delayed in iNOS ko mice and wild-type litter mates treated with a continuous infusion of a partially selective iNOS inhibitor.[60] Furthermore, adenoviral-mediated iNOS gene transfection of iNOS ko animals reverses the delayed wound closure.[60] However, the quality of incisional wound healing is not significantly different between wild-type and iNOS ko mice.[61] This puzzling phenomenon may be explained by the finding that expression of TGF-β1 and eNOS is increased while that of basic fibroblast growth factor (bFGF) and IL-4 are reduced in iNOS ko incisional wounds.[61] Because the epithelial-stimulatory properties of IL-4 combined with the fibrogenic and angiogenic potential of bFGF are particularly important in excisional wounds, it was proposed that their reduced expression impaired excisional healing in iNOS ko mice.[60,61] Meanwhile, compensation by increased TGF-β1 and eNOS expression explained the persistence of normal incisional wound healing in iNOS ko mice.[60] These compensatory mechanisms would not have time to develop in the acutely iNOS inhibited animals treated with NOS inhibitors mentioned in the studies above.[8,56,58,62]

Relative NOS isoform activity in healing wounds

By investigating the time course of NO expression in the healing wound, Lee et al.[63] found a maximal NOS activity at 24 hours, followed by a steady decline in NOS activity over the next 5–10 days. Lee et al. demonstrated sustained

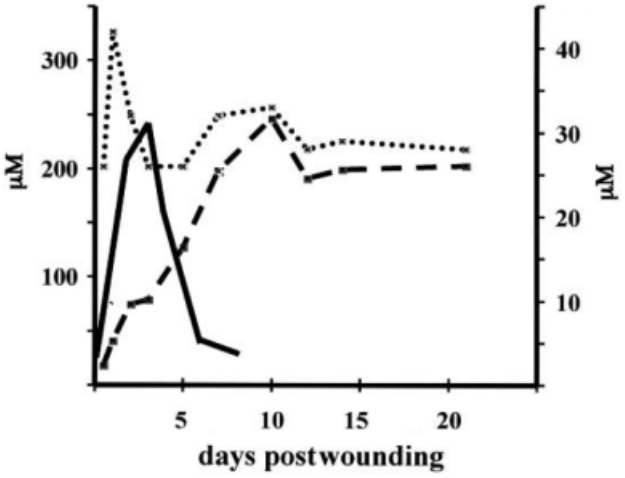

Figure 2. Time course of NO by-products (citrulline, ornithine, and NO2) in wound fluid. Ornithine: dashed line (----); NO_2: solid line (—); Citrulline: dotted line (.....). With permission from Rizk et al.[67]

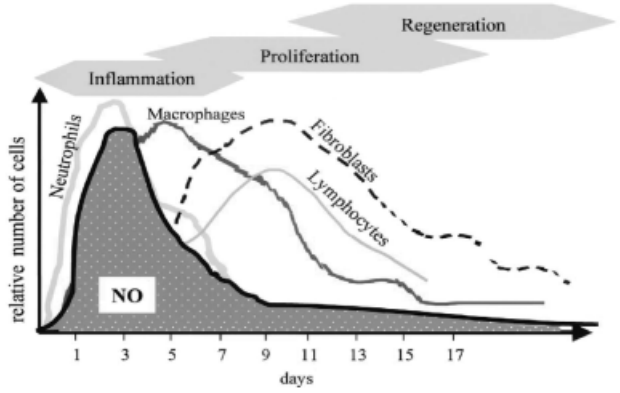

Figure 3. Phases of wound healing and the generation of wound NO. With permission from Witte and Barbul.[7]

NOS activity up to 10 days postwounding. Other studies confirm maximal iNOS expression in cutaneous wounds 1–5 days postwounding.[64] Gene expression for iNOS paralleled the upsurge in enzymatic activity, with an initial surge at 24 hours postwounding, which was followed by continuous but low-level expression for 10 days postwounding. The high concentrations of NO produced in the acute inflammatory phase following wounding suggest a role for NO in the acute nonspecific host response. This could be as part of the initial lines of defense in the wound, while exposure to microbial invasion would be at its highest. Studies on rat colonic anastomoses seem to support this concept. Thornton et al.[58,65] showed that NOS activity peaked at 24 hours postcolonic anastomosis. Furthermore, immunohistochemistry of normal anastomotic crosssections showed a concentration of iNOS-2 positive cells along the mucosal edge at 24 hours postanastomosis. This indicated an increase in NO production in this region, where antimicrobial challenge is the highest.[58] It should be noted that species-specific differences in the kinetics of NO formation have been shown.[66] The overall time course of iNOS activity and NO production during the wound-healing process should be viewed as a decreasing curve over time[67] (Figures 2 and 3).

Cellular contributions to NO production

As mentioned earlier, many of the cells involved in the wound healing process are capable of producing NO through the activity of either a constitutive NOS or iNOS. Such cells include macrophages (iNOS),[68] keratinocytes (eNOS and iNOS),[69–71] endothelial cells (eNOS),[72–74] and fibroblasts (eNOS and iNOS).[73,75–77] The majority of NO produced in the early inflammatory stages of wound healing is likely because of recruited proinflammatory cells, especially macrophages.[68] Also implicated in NO production, although to a lesser degree, are fibroblasts, keratinocytes, and endothelial cells.

NO EFFECTS IN WOUND HEALING

The primary effects of NO would appear to be particularly useful to the initial (inflammatory) phase of wound healing. These effects include vascular permeability changes

and vasodilation,[78–81] antimicrobial activity,[82–85] and antiplatelet aggregation activity.[86] The expression of iNOS has been shown to peak at around 48 hours postwounding, which suggests that its predominant activity is during the inflammatory phase.[43] NO modulates both chemoattractant cytokines that initiate postwound inflammation (including IL-8,[87] TGF-β1[88]) and the chemotaxis of neutrophils and monocytes.[89] IL-8 decreases the expression of iNOS in neutrophils.[90] TGF-β suppresses NO production while NO activates latent TGF-β1.[91] These cells are very active in the production of important cytokines and produce, among other products, tumor necrosis factor (TNF)-α.[92–94] This production of TNF-α is also regulated by NO via a cGMP-independent mechanism in human peripheral blood monocytes.[95]

It has been suggested that the presence of NO helps the transition of the wound from the inflammatory phase to the proliferative phase of wound healing.[96,97] In support of this concept, the monocyte-attracting chemotactic cytokine RANTES (regulated upon activation, normal T-cell expressed and secreted) is downregulated by NO both in vitro and in vivo.[96] Another mechanism supporting this transitionary role for NO in wound healing is the decrease in production of monocyte-attracting macrophage chemoattractant protein-1 (MCP-1) by wound edge hyperproliferative keratinocytes.[97] This effect of NO during the inflammatory phase of wound healing could help to push the process forward. So, with an important role in the initiation of the inflammatory cascade and subsequent processes, and a probable role in concluding the inflammatory aspects of wound healing, the evidence for a temporal role of NO in wound healing is convincing.

Proliferation, differentiation, and apoptosis

The effects of NO on cellular proliferation seem to depend largely on local concentrations. In keratinocytes, the selective iNOS inhibitor L-N6-(1-iminoethyl)lysine decreases proliferation at the wound edge while low doses of NO increased keratinocyte proliferation in vitro.[98] Keratinocyte proliferation is also increased in vitro by 8-bromo-cGMP,[99] which is an analog of NO second messenger cGMP. Interestingly, NO, at higher doses, has been found to inhibit keratinocyte proliferation.[100] In an example of local feedback regulation, the cytokines that promote iNOS expression by keratinocytes, IFN-γ, LPS, and TNF-α, all inhibit the actual growth of these cells.[101] Epidermal growth factor (EGF) promotes keratinocyte proliferation and blocks keratinocyte-derived production of NO.[100,101] NO donors (sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine enhance bFGF and platelet-derived growth factor-induced DNA synthesis in human dermal fibroblasts at low concentrations but higher concentrations reduced or negated the responses.[102] This was mediated by NO-stimulated cyclicGMP as the second messenger analog 8-bromo-cGMP had similar effects.[102] Cells may have an NO sensitivity threshold below which they differentiate and proliferate, and above which growth is inhibited. For example, proliferation of fibroblasts and smooth muscle cells is inhibited by NO, even at low levels, while similarly low concentrations of NO stimulate proliferation of endothelial cells and keratinocytes and higher concentrations of NO are required in in vitro models to inhibit the proliferation of these cells.[103,104] NO protects against ultraviolet B radiation-induced keratinocyte apoptosis in vitro.[105] In another study where NO suppression of ultraviolet B radiation-induced apoptosis of keratinocytes was investigated, mice null for nNOS exhibited significantly higher apoptosis than wild-type mice both in the dermis and epidermis while mice null for iNOS exhibited more apoptosis than wild-type mice in the dermis only.[106] Other protective effects of NO against apoptosis have been shown in endothelial cells[107] in which NO mediates vascular endothelial growth factor (VEGF)-induced proliferation.[108,109]

Angiogenesis and the dynamic interplay between VEGF and NO

The process of new microvessel formation (angiogenesis) is of central importance in the process of wound healing and NO plays a pivotal role.[110] NO increases angiogenesis in ischemic murine tissues.[111] NOS inhibitors suppress capillary organization in vitro[108] and impair granulation tissue angiogenesis in gastric ulcers.[112,113] Meanwhile, VEGF increases NO production through the up-regulation of eNOS[72,73,114–116] and iNOS may be equally important.[117] VEGF promotes endothelial cell proliferation and mediates the activation of mitogen-activating protein kinase via NO signaling.[118–120] Lee et al.[121] used an eNOS ko mouse model to demonstrate that the loss of this isoform led to decreased angiogenesis and, as a result, inhibited wound healing. Mice deficient in eNOS have impaired angiogenesis that is not improved by VEGF administration.[122,123] suggesting that NO is a downstream mediator for VEGF-induced angiogenesis. Other VEGF-related processes dependent on NO include endothelial cell migration, decreased cellular adhesion, and organization.[99,101,109,118,124,125] Cytokine-stimulated keratinocytes are a major source of VEGF during cutaneous wound healing.[22,126] NO mediates this VEGF expression/synthesis and iNOS inhibitors block in vitro and in vivo production.[22,122,123,126,127] A negative feedback mechanism appears important in the regulation of VEGF availability. After balloon catheter-induced endothelial injury, NO donors downregulate protein kinase C-induced VEGF expression in local vascular smooth muscle cells by interfering with the binding of the transcription factor activator protein-1.[128] This suggests that in vivo, NO secreted by a restored endothelium can function as part of a negative feedback mechanism, down-regulating VEGF to basal levels. NO is also an important factor in VEGF-independent monocyte-induced angiogenesis,[129] which requires substance-P[130] and TGF-β1.[131]

Collagen deposition, remodeling, and maturation

There is a strong relationship between the availability of NO in the healing wound and the quality of subsequent collagen deposition.[8,44,56,59,132] Both wound-derived and normal skin-derived fibroblasts produce increased levels of collagen after treatment with an NO donor *in vitro*.[103,133] Similarly, such collagen production is reduced after NOS inhibition.[103,133]. As mentioned previously, iNOS[59] and

eNOS ko[121] mice display impaired wound closure, while excisional wound contraction is delayed by pharmacological iNOS inhibition.[60,121,134,135]

NO MECHANISMS OF ACTION

NO acts as a signaling molecule via the second messenger cGMP[136] or by cGMP-independent[103] effects on gene transcription and translation.[136–144] Either way, NO tightly regulates cell growth via stimulatory and inhibitory mechanisms under certain conditions. Negative effects on proliferation may be related to ornithine decarboxylase inhibition, an enzyme that catalyzes the formation of putrescine and a rate-limiting step in polyamine production (Figure 1).[144] Other cytostatic and cytotoxic effects of NO are mediated via inhibition of target enzymes such as cytochromes, mitochondrial aconitase (Krebs Cycle), NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase (complexes I and II of the mitochondrial electron transport chain), and ribonucleotide reductase (DNA synthesis).[137,138] Cells affected in this way include endothelial cells, smooth muscle cells, hepatocytes, and fibroblasts.[139–141] NO regulates gene expression by nitrosylation of the thiol-binding site of nuclear factor κβ in a feedback-inhibitory mechanism that prevents binding of the transcription factor to the iNOS promoter.[66,142] With the amplification of other regulatory mechanisms, NO regulates gene expression through calcium and protein kinase A-dependent activation of another transcription factor CREB.[145] Because it engages in posttranslational collagen regulation via protein kinase C activity,[146,147] NO may downregulate collagen synthesis in fibroblasts under some conditions. While it induces apoptosis via p53 mechanisms in some tumor cells, NO also inhibits activation of pro-apoptotic proteases (caspases) in healthy cells.[67,139,143] This reflects both concentration- and cell type-dependent effects of NO under variable circumstances but in general, tonic low concentration NO is protective while higher burst production is pro-apoptotic.

NO IN DIABETES-IMPAIRED WOUND HEALING

Many impaired wound healing states have been associated with depleted NO production. Protein-calorie malnutrition results in reduced levels of both nitrites and nitrates within wound fluid.[55] Corticosteroids are well known for their deleterious effects on the wound healing process,[57] and they are also known to be potent inhibitors of iNOS.[64] There are strong correlations between reduced cutaneous NO availability and well-documented impairments in diabetic wound healing. In diabetes, the complex overall process of wound healing, and its constituent phases are all impaired. Early in diabetic wound healing, impairment of chemotaxis, phagocytosis, and depleted levels of local antioxidant levels[148–150] result in reduced bacterial killing. Later in diabetic wound healing, local growth factor concentrations are reduced,[151,152] local glucocorticoid concentrations are elevated,[153] cellular proliferation is inhibited[154,155] and there is an up-regulation of apoptosis.[156] The excessive adiposity of diabetes may also play an inhibitory role in diabetic wound healing.[157] Normalization of blood glucose concentrations may help to reverse some of the impaired mechanisms of wound healing, e.g., the processes of collagen metabolism and subsequent cross-linking are benefited by treatment of diabetes with insulin.[158,159]

The diabetic state is characterized by greatly reduced expression of NOS and a resultant reduction in availability of NO in the cutaneous wound environment.[8–11] Luo et al.[10] suggested that the augmentation of cutaneous eNOS protein expression and constitutive NOS activity observed in normal animals in response to wounding are absent in type 1 diabetic mice. Furthermore, cutaneous gene therapy with eNOS accelerated the wound healing rate observed in streptozotocin (STZ), induced diabetic mice.[10] Witte et al.[160] confirmed that diabetic wound healing is characterized by a nitric oxide-deficient state characterized by reductions in wound breaking strength and collagen deposition and a severely impaired inflammatory process when compared with controls. It was shown that treatment with molsidomine (*N*-ethoxycarbomyl-3-morpholinyl-sidnonimine), a nitric oxide donor, significantly improved fresh wound breaking strength and wound collagen content in diabetic animals.[160] Improved collagen remodeling capability was also shown in the wounds of these molsidomine-treated animals, reflected by an increase in activity of matrix metalloproteinase-2.[160] In another study on STZ-induced diabetes, NO releasing polyvinyl alcohol, hydrogel dressings were shown to reverse partially the diabetes-induced impairments to wound healing.[161]

Hyperglycemia is known to decrease the endothelial production of NO,[162,163] and simultaneously induces a series of cellular events that increase the production of active oxygen species, which in turn rapidly inactivate NO and form peroxynitrite. Such active oxygen species include superoxide anion.[164,165] Glucose concentration dependently increases superoxide levels in normal mouse skin and there is a marked increase of cutaneous superoxide levels in STZ-induced type 1 diabetic mice.[10] The mechanisms through which hyperglycemia induces these reactive oxygen species are unclear but may be related to activation of the mitochondrial electron transport chain.[165] Once formed, peroxynitrite further inhibits NO availability through oxidation of the NOS co-factor tetrahydrobiopterin,[166,167] resulting in enzyme uncoupling and a preferential increase in superoxide anion production in favor of NO production.[168] Increasing superoxide concentrations lead to a state of oxidative stress, mediating a condition of endothelial cellular dysfunction and leading to an overall state of cardiovascular dysfunction.[169] This is supported by the observation that intra-arterial infusion of ascorbic acid, a water-soluble antioxidant capable of scavenging superoxide anion, restores endothelium-dependent vasodilation in healthy subjects exposed to hyperglycemic clamp, and in patients with type 1 or 2 diabetes.[164,170,171]

Insulin resistance, as in type 2 diabetes mellitus, has a negative effect on the availability of NO. Insulin stimulates NO production in endothelial cells by increasing the activity of NOS via activation of phosphatidylinositol-3-kinase and akt kinase.[172–174] Thus, in healthy subjects an important role for insulin is that of that of promoting endothelium-dependent, i.e., NO-mediated, vasodilation. Hence,

in insulin-resistant subjects, endothelium-dependent vasodilation is reduced.[175] Furthermore, Mather et al.[176] showed that insulin-mediated glucose disposal correlated inversely with the severity of the impairment in endothelium-dependent vasodilation.

In summary, NO regulates some of the most important facets of the delicately balanced systems that govern wound healing. Derangements in the availability of NO explain many of the poor wound healing characteristics found in diabetes. Further work is required to establish all the functions of NO in wound healing such that clinical applications of this knowledge may be delivered.

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