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Nitric Oxide–Containing Nanoparticles as an Antimicrobial Agent and Enhancer of Wound Healing

Richard B. Weller[1]

Nitric oxide (NO) is known to be involved in wound healing and to have antimicrobial actions. Developing a means of delivering a diatomic, gaseous free radical has been a technical challenge. Using a combination of techniques, Martinez and colleagues have developed an ingenious method of storing NO, and they demonstrate its efficacy in treating infected wounds.

Journal of Investigative Dermatology (2009) **129**, 2335–2337. doi:10.1038/jid.2009.149

Nitric oxide (NO) biology has been a subject of intense study over the past 15 years, but clinical applications have lagged behind our growing understanding of its varied biological roles. First described as endothelium-derived relaxant factor, the elucidation of the mechanism by which this soluble, short-half-life mediator is released from endothelial cells to dilate adjacent vascular smooth muscles via the cyclic guanosine monophosphate (cGMP) and its subsequent identification as NO earned a Nobel Prize in Medicine for Drs. Furchgott, Ignarro, and Murad in 1998.

NO has a far wider range of actions than simply vasodilatation, and the discovery that carbon monoxide is biologically active indicates it may be the prototype of a new class of small free-radical messengers. These diatomic free radicals interact with enzymes, particularly those containing transition metals, to activate or inhibit them. NO is produced by a family of three NO synthases (NOSs), with arginine as the substrate. Endothelial and neuronal NOSs are constitutively expressed, whereas inducible NOSs (iNOSs) release much higher concentrations of NO in response to a number of cell-specific stimuli. Nonenzymatic production of NO by the reduction of nitrite and nitrosothiols, in particular, also occurs. Both enzymatic and nonenzymatic mechanisms of production occur in or on the skin. NO plays a part in keratinocyte growth and differentiation, apoptosis regulation, platelet aggregation, neurotransmission, control of oxygen delivery, and vasodilatation. Aberrant regulation of NO is a feature of septic shock and pulmonary hypertension, as well as inflammatory conditions such as psoriasis and rheumatoid arthritis.

NO as an anti-infective agent

NO is important in the innate immune system (Fang, 2004). Stimulation of microbial pattern recognition receptors in phagocytic cells leads to iNOS transcription and high-output NO production. NO can interfere directly with DNA replication and cell respiration through the inactivation of zinc metalloproteins, but it more commonly interacts with simultaneously produced reactive oxygen species to generate an array of reactive nitrogen intermediates. This allows finely regulated production of a spatially and temporally restricted array of antimicrobial effector molecules acting on different targets within the microbial cell. NO donors have demonstrated promise as antimicrobial agents, but in most of these systems NO is reported to act in combination with other agents (Weller *et al*., 2001; Lopez-Jaramillo *et al*., 1998). Its efficacy as an independent antimicrobial has been less well defined.

NO and wound healing

NO is produced by healing wounds, and its known roles in angiogenesis, collagen deposition, and keratinocyte proliferation all indicate that its production is an important part of the wound healing cascade. Experiments demonstrating that pharmacological or genetic (NOS knockout) reduction of NO impairs the speed and effectiveness of wound healing and that this process can be reversed by restoring NO production with increased NOS substrate (arginine) or by transfecting with the missing NOS gene (Yamasaki *et al*., 1998) confirm this suggestion. Furthermore, a number of nitric oxide donors have exhibited benefit in experimental wound models (Weller and Finnen, 2006; Luo and Chen, 2005).

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NO donors

Devising a means of storing a small, gaseous free radical has been a considerable technical challenge, requiring a different approach from the mechanism of currently available pharmaceuticals, which are complex molecules with action and specificity that rely on their three-dimensional shape and receptor–agonist interactions. NO has a short half-life and high reactivity and, being lipophilic, readily crosses cell walls and diffuses rapidly down a concentration gradient from its site of production. Specificity of action relies on the ability of NO to be either produced or released at the required site and at a rate high enough to generate a concentration gradient. The ideal donor drug would store NO stably at room temperature for an extended time and release it consistently when required at the pharmacologically specified dose and with a long enough duration to exert its biological action. The agent should be nontoxic and noninflammatory. Several NO donors have been developed to meet these criteria, and the accessibility of the skin makes it an ideal organ in which to develop NO donor-based treatments.

At the simplest level, inhaled gaseous NO has been used to treat pulmonary hypertension and respiratory distress syndrome, but a gaseous form would be impractical for skin disease. NO-containing compounds such as nitrosothiols, NO-binding organic molecules, and nitrosated thiosugars, which will break down in contact with the skin, have been tested in humans. Nitrosothiols (–SNOs) are formed by the reaction of NO with thiol groups, and they are biologically active. In addition, the nitrosothiol bond is relatively unstable and liberates NO in the presence of ultraviolet radiation, copper, or mercury. Direct transnitrosation reactions can also occur with adjacent thiol groups, which are plentiful in the cysteine-rich stratum corneum and may prolong the actions of nitrosothiol. Nitrosated thiosugars (Khan *et al*., 2003) undergo thermal decomposition.

A second approach to delivering NO is to use a more stable precursor, which can be chemically activated at the time of application. Two-part creams in which nitrite is mixed with thiols can be used to form nitrosothiols at the time of application. Alternatively, nitrite can be mixed with an acid such as ascorbate, leading to the reduction of nitrite and the release of NO:

$$NO_2^- + H^+ \leftrightarrow HNO_2$$
$$2HNO_2 \leftrightarrow N_2O_3 + H_2O$$
$$N_2O_3 + NO\bullet + NO_2$$

The chemistry is more complex than this simple equation illustrates, however, and a number of reactive nitrogen intermediates are also released, depending on the acid used. Acidified nitrite has been used successfully to treat cutaneous fungal, leishmanial, and mycobacterial infections (Fang, 2004), and it hastens wound healing in normal and diabetic mice (Weller and Finnen, 2006). The precise biological effects of acidified nitrite creams depend on the p*K*a and type of acid used as a reducing agent, but they may cause inflammation. Placing a semipermeable membrane between the skin surface and mixed creams mitigates the inflammatory effects of acidified nitrite, while allowing NO delivery (Hardwick *et al*., 2001). The undoubted antimicrobial efficacy of acidified nitrite-based treatments may rely on the range of nitrogen oxides produced, in addition to NO.

Finally, local NO storage and delivery are possible in nanoporous materials. Zeolites, which can contain large quantities of NO reversibly constrained within their lattice structure, are the prototypic microporous material. Structural alterations in pore size and chemically bound metal ions within the zeolite allow modulation of the rate and extent of NO release (Mowbray *et al*., 2008). Exposure to moisture, such as that found on the skin surface, triggers NO release as water enters the lattice structure and exchanges for NO.

Martinez *et al*. (2009, this issue) describe a novel NO-releasing cream that uses a combination of these methods. The same group previously developed a hydrogel/glass composite that is synthesized using tetramethylorthosilicate, polyethylene glycol, and chitosan to form the NO-bearing composite, and nitrite and glucose to form NO (Friedman *et al*., 2008). During the preparation process, the mixture is heated to 70 ºC, under which conditions glucose reduces nitrite to release NO, which remains trapped within the composite structure. The resulting composite is milled and desiccated to form nanoparticles from which NO is released upon exposure to water. The rate of release can be controlled by modifying the size of polyethylene glycol molecules, and the amount of NO loaded into the nanoparticles can be determined by the initial nitrite concentration.

> Creams that release NO may benefit wound healing and inhibit infection

A number of previous studies have demonstrated reductions in wound healing times with NO donor treatment (Luo and Chen, 2005; Weller and Finnen, 2006), but this is the first study in which such a donor exhibits simultaneous wound healing and antimicrobial benefits. Methicillin-resistant *Staphylococcus aureus* and the increased incidence of chronic leg ulceration in an aging population are significant public health problems. The advent of a technology that may benefit both—individually or jointly—seems promising. The NO released by the nanoparticles described by Martinez *et al.* may be solely responsible for their antimicrobial properties, in which case other topical NO donors would be expected to have similar effects. Alternatively, there may be synergy between NO and the chitosan in the nanoparticle composite.

A considerable body of evidence suggests that topical NO donors improve wound healing, and a number of potential agents have demonstrated efficacy in animal models. Clinical trials are needed to determine which of these agents is most likely to be effective in humans. The timing of application of a NO donor after wounding will probably be an important variable. Previous murine experiments have shown that NO donors are effective in improving wound healing only when application starts 2 to 4 days after injury (Weller and Finnen, 2006). This indicates

that additional NO is beneficial only at certain phases in the wound healing cascade, and different wounds may require different NO donors. Infected wounds would be an obvious target for nanoparticle NO (Martinez *et al.*, 2009), whereas other NO donors have exhibited efficacy at healing wounds in diabetic mice. Somewhat counterintuitively, the relatively large number of NO donors shown to improve wound healing seems to have slowed the clinical development of this family of drugs. The ubiquity and simplicity of NO have produced a complex intellectual property environment in which the pharmaceutical industry, the government, and charitable donors are unwilling to fund clinical trials lest they lose the commercial benefits resulting from a successful outcome to a patent holder with a competing claim. One hopes that such concerns will not hinder the clinical development of this promising line of therapies.

CONFLICT OF INTEREST

Richard B. Weller has advised a number of companies on the development of NO donors for clinical applications.

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Spitting Image: Tick Saliva Assists the Causative Agent of Lyme Disease in Evading Host Skin's Innate Immune Response

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Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is transmitted through ticks. Inhibition of host skin's innate immune response might be instrumental to both tick feeding and *B. burgdorferi* transmission. The article by Marchal *et al.* describes how tick saliva suppresses *B. burgdorferi*–induced antimicrobial peptide production. This inhibition directly facilitates survival of the spirochete and might lead to diminished chemotaxis of leukocytes toward the site of the tick bite.

Journal of Investigative Dermatology (2009) **129**, 2337–2339. doi:10.1038/jid.2009.202

Lyme disease, or Lyme borreliosis, was first recognized as a distinct clinical entity in 1975 in Old Lyme, Connecticut, in children at the Yale–New Haven Hospital who were initially thought to have juvenile rheumatoid arthritis (Steere *et al.*, 1977). Previously, however, certain clinical signs of the disease had been described in Europe (Afzelius, 1921). *Ixodes scapularis*, *Ixodes ricinus*, and *Ixodes persulcatus* are the most important vectors for Lyme borreliosis in the United States, Europe, and Asia, respectively, and the disease is caused by spirochetes of the *Borrelia burgdorferi* sensu lato group (Wang *et al.*, 1999). In Europe and Asia, three major *Borrelia* genospecies (*B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*) are the causative agents. By contrast, only *B. burgdorferi* sensu stricto strains are present in the United States. The obligate enzootic life cycle of the spirochetes involves ticks, primarily *Ixodes* ticks, and a variety of vertebrate hosts, including small rodents, large mammals, and birds (Anderson and Magnarelli, 1980). In general, uninfected tick larvae acquire the bacterium by feeding on infected animals. Ticks remain infected during their consecutive molting periods, enabling both nymphal and adult ticks to transmit spirochetes to (larger) animals and humans.

To secure attachment of the tick and to ensure susceptibility of reservoir hosts for future tick infestations, tick saliva contains modulators of host immune responses. During the course of a blood meal, which can take up to seven days, ticks introduce saliva containing a wide range of physiologically active components. Immunosuppressive proteins in tick saliva interfere with

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