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Dermal Nitrite Application Enhances Global Nitric Oxide Availability: New Therapeutic Potential for Immunomodulation?

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TO THE EDITOR

Systemic administration of nitrite (NO2-) affords protection against ischemia/reperfusion-related organ damage (Lundberg et al., 2008), whereas topical nitrite application exerts local antimicrobial effects and promotes wound healing (Weller et al., 2001; Weller and Finnen, 2006). The latter two actions require acidification, are often accompanied by increased blood flow (Tucker et al., 1999; Gribbe et al., 2008), and are thought to be mediated by the formation of nitric oxide (NO), albeit not all effects of nitrite necessarily occur through NO generation (Bryan et al., 2005). Although the significance of NO in cutaneous physiology is widely recognized (Bruch-Gerharz et al., 1998; Weller, 2003), the relevance and biological role of its oxidation product, nitrite, are incompletely understood (Suschek et al., 2006), as is the mechanism by which dermal nitrite couples to systemic NO/nitrite physiology.

Nitrite concentrations in human skin are considerably higher than those in circulation (Paunel et al., 2005; Mowbray et al., 2009) and are a reflection of local NO synthase activity. Nitrite is also a constituent of human sweat, possibly produced via reduction of nitrate by commensal heterotrophic bacteria colonizing the skin (Weller et al., 1996). The actions of nitrite in or on the skin have been proposed to contribute to host defense against skin pathogens and include modulation of cutaneous T-cell function, keratinocyte differentiation, blood flow, and protection against UV-radiation damage (Weller et al., 1996; Suschek et al., 2006). Whether these actions of nitrite extend beyond the skin is unknown. Recently, nitrite production via autotrophic oxidation of ammonia by dermal microflora has been proposed to contribute to bodily NO status and immune system function (Whitlock and Feelisch, 2009). An important prerequisite for the validity of this concept is nitrite’s ability to permeate the skin. Although substances with a molecular weight of <500 Da are believed to cross the stratum corneum with ease (Bos and Meinardi, 2000), the same rules may not apply for negatively charged ions (nitrous acid (HNO2) has a pH of 3.4 and is thus largely (> 99.9%) dissociated at physiological pH; Butler and Ridd, 2004). Apart from anecdotal evidence of fatal methemoglobinemia after application of a liniment containing large quantities of nitrite (Saito et al., 1996, 1997), no information on dermal nitrite uptake is available. We therefore sought to investigate, using a rodent model, whether nitrite permeates the skin to reach internal organs through the circulation.

Pharmacologically relevant doses of nitrite (0.1, 1.0, and 10 mg kg−1 in phosphate-buffered saline; pH 7.4) or vehicle were topically applied in equal volumes (800 µl) to a shaved area of dorsal skin, and concentrations of several NO-related metabolites (nitrite, nitrate, S- and N-nitroso compounds (RXNO), and NO-heme species) were determined simultaneously in multiple compartments using extensively validated ion-chromatographic and gas-phase chemiluminescence-based techniques (Feelisch et al., 2002; Rodriguez et al., 2003; Bryan et al., 2004) (see Supplementary Materials for details). We find (i) that no acidification is required to increase NO-related metabolites in blood and tissues and (ii) that compartments in which increases occur include those important for priming and functional modulation of immune cells.
Because all the NO-related metabolites determined either can be metabolized to NO or are endowed with NO-like bioactivities, dermal nitrite application would seem to lead to global increases in NO availability, with consequent modulation of organ and immune cell function. In support of this notion, nitrite was found to potently inhibit T-cell and other inflammatory cell accumulation in the lung and airway secretion of IL-13 in a murine model of allergic asthma. Although nitrite penetration through skin per se is perhaps not surprising, the speed and efficacy with which this process occurred were unexpected. Dose-proportional increases in NO-related metabolites were monitored in three representative compartments (plasma, heart, and liver) and became evident within minutes of nitrite application, with concentrations peaking within 5–15 minutes, depending on the compartment under study (Figure 1, inset(s)). Maximal concentrations of NO-related metabolites were three- to sixfold lower after dermal application, compared with intraperitoneal administration of equivalent doses (Bryan et al., 2005), but no effort was undertaken to directly compare rates and the extent of uptake between application routes. Topical administration of nitrite-free vehicle did not significantly change basal levels (data not shown). Figure 1 depicts the effects of 10 mg kg\(^{-1}\) nitrite at 15 minutes after application. Marked increases in nitrite, nitrate, RXNO, and NO-heme species were observed in most, but not all, compartments. Notable exceptions were kidney (nitrite), brain...
Dermal Nitrite Administration

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Cells oxidize NO to nitrite, and nitrite can be reduced back to NO under certain conditions, yet the biology of these two species is not interchangeable and actions of nitrite cannot be simply inferred from known effects of NO. We therefore sought to obtain direct evidence for immunomodulation by nitrite in T-cell receptor transgenic mice challenged with aerosolized ovalbumin. Using this model of allergic asthma (Wilder et al., 2001), we found that nitrite (10 mg l\(^{-1}\) administered for 4 days with drinking water) potently inhibits the number of T lymphocytes and other inflammatory cells, as well as the production of the Th2-derived effector cytokine, IL-13, in bronchial alveolar lavage fluid (Supplementary Figure 1).

Collectively, our findings suggest that dermal nitrite administration provides a simple, noninvasive alternative to other application forms that can be conveniently used for percutaneous systemic therapy (presumably with lower risk of N-nitrosamine formation compared with oral application). Our results may also have important physiological implications inasmuch as sweat-derived nitrite, from nitrate reduction or ammonia oxidation, seems to have the potential to affect immune responses through modulation of NO availability. Collectively, these observations may shift the current emphasis of nitrite from cardiovascular pharmacology to immunology. Further investigations are warranted to confirm our findings and explore their therapeutic potential.

**CONFLICT OF INTEREST**

DRW has patented the use of topical ammonia-oxidizing bacteria as a health treatment to prevent and treat disorders characterized by low NO/NOx status. The other authors state no conflict of interest.

**ACKNOWLEDGMENTS**

We thank Nathan S Bryan and Selena Bauer for their assistance in preliminary experiments related to this work, Margaret Delano for advice on lymph node harvest, and Lia Cross for help with T-cell isolation, staining, and FACS analysis. This project was supported by grants from the US National Institutes of Health (DA020644 and HL69029 to MF), the UK Medical Research Council (MRC Strategic Appointment Scheme to MF), and a Kirschstein US National Research Service Award Cardiovascular Training grant (HL07224 to BOF).

**Table 1. NO-related metabolites in immune-relevant compartments 15 minutes after dermal application of 10 mg kg\(^{-1}\) sodium nitrite (in PBS, pH 7.4) compared with effects of vehicle (PBS) alone**

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Vehicle ((n=5))</th>
<th>Nitrite ((n=3))</th>
</tr>
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<tbody>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.07 ± 0.04</td>
<td>3.36 ± 1.33*</td>
</tr>
<tr>
<td>Nitrate</td>
<td>2.96 ± 0.64</td>
<td>5.13 ± 1.56</td>
</tr>
<tr>
<td>RXNO</td>
<td>0.24 ± 0.06</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>NO-heme</td>
<td>4.34 ± 2.97</td>
<td>11.6 ± 1.9</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>&lt;0.04</td>
<td>1.44 ± 0.57*</td>
</tr>
<tr>
<td>Nitrate</td>
<td>1.17 ± 0.15</td>
<td>5.92 ± 1.00**</td>
</tr>
<tr>
<td>RXNO</td>
<td>0.09 ± 0.01</td>
<td>0.99 ± 0.46*</td>
</tr>
<tr>
<td>NO-heme</td>
<td>1.24 ± 0.85</td>
<td>12.2 ± 5.86*</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>3.71 ± 1.29</td>
<td>8.53 ± 1.91</td>
</tr>
<tr>
<td>Nitrate</td>
<td>40.78 ± 25.29</td>
<td>84.25 ± 42.29</td>
</tr>
<tr>
<td>RXNO</td>
<td>0.08 ± 0.05</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>NO-heme</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

NO, nitric oxide; PBS, phosphate-buffered saline; RXNO, S- and N-nitroso compounds. The compartment denoted as “lymph nodes” is a homogenate of pooled superficial cervical, axillary, and iliac lymph nodes (ND, not determined owing to limited sample availability). Data are means ± SEM from \(n\) individual experiments and expressed as nmoles per gram wet weight (for nitrite, nitrate, and RXNO) and pmoles per gram wet weight for NO-heme products; *\(P<0.05\), **\(P<0.01\) versus vehicle.

Although there is precedence for increased NO-related metabolites in blood and tissues after systemic nitrite administration (Bryan et al., 2005), information regarding its effects on the immune system is scarce (Ustyugova et al., 2002; Cape and Hurst, 2009). Lymph nodes from vehicle-treated animals showed remarkably high nitrite/nitrate concentrations, indicative of high NO production rates under basal conditions (Table 1). Dermal nitrite application (10 mg kg\(^{-1}\)) induced widespread changes in NO-related metabolites within thymus, spleen, and peripheral lymph nodes, depicting clear increases for most—except RXNO, which decreased in thymus and lymph nodes. Preliminary analysis of the CD3-positive cell fraction of lymph nodes confirmed that, whereas intracellular nitrite and nitrate concentrations double, S-nitrosation of T cells drops by 45–60% shortly after dermal nitrite application. Little is known about the effects of changes in protein nitrosation on immune cell activity, but various functional consequences are likely, given the importance of redox-based signaling (Janssen-Heininger et al., 2008) and the versatility of NO in regulating T-cell, dendritic cell, and macrophage function in particular and immune responses in general (Bogdan, 2001; Niedbala et al., 2006; Mowbray et al., 2008).
SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES