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

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Abstract

Nitrite in/on the skin has potent antimicrobial effects and is believed to act as a local nitric oxide (NO) reservoir capable of modulating dermal cell function. Using male Wistar rats we here show that nitrite, when applied in neutral aqueous solution to the skin surface, readily permeates the dermis to reach the systemic circulation and cause widespread nitros(yl)ation of tissue proteins. These events extend to all internal organs investigated including compartments crucial for the regulation of immune cell function such as thymus, spleen and lymph nodes. Using a transgenic mouse model of allergic asthma, we further find that systemic nitrite administration potently suppresses the number of T-cells and other inflammatory cells and the production of the Th2 effector cytokine, IL-13 in the lung. Thus, nitrite formed via reduction of nitrate or oxidation of ammonia on the skin, may exert previously unappreciated systemic effects akin to NO. These observations have the potential to shift the current emphasis of nitrite from cardiovascular pharmacology to immunology.

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TO THE EDITOR

Whereas systemic administration of nitrite (NO_2^-) affords protection against ischemia/reperfusion-related organ damage (Lundberg *et al.*, 2008), topical nitrite application exerts local antimicrobial effects and promotes wound healing (Weller *et al.*, 2001; 2006). The latter two actions require acidification, are often accompanied by increased blood flow (Tucker *et al.*, 1999; Gribbe *et al.*, 2008) and 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). While the significance of NO in cutaneous physiology is widely recognized (Bruch-Gerharz *et al.*, 1998; Weller, 2003) relevance and biological function of its oxidation product, nitrite, are incompletely understood (Suschek *et al.*, 2006), as is how 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 a reflection of local nitric oxide synthase activity. Nitrite is also a constituent of human sweat, possibly from reduction of nitrate by commensal heterotrophic bacteria colonizing the skin (Weller et al., 1996). The actions of nitrite in/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 action of nitrite extends beyond the skin is unknown. Recently, nitrite production via autotrophic oxidation of ammonia by the dermal microflora has been proposed to contribute to bodily NO status and immune system function (Whitlock & Feelisch, 2009). An important prerequisite for the validity of this concept is nitrite's ability to permeate the skin. While substances with a molecular weight of <500 Dalton are believed to cross the stratum corneum with ease (Bos & Meinardi, 2000), the same rules may not apply for

negatively-charged ions [1]. Apart from anecdotal evidence of fatal methemoglobinemia following 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 via the circulation.

Pharmacologically relevant doses of nitrite (0.1, 1.0, and 10 mg·kg-1 in PBS; pH=7.4) or vehicle were topically applied in equal volumes (800 μL) to a shaved area of the dorsal skin, and concentrations of several NO-related metabolites (nitrite, nitrate, S- and N-nitroso (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 **Online-Supplement** for details). We find i) that no acidification is required to increase NO-related metabolites in blood and tissues, and ii) that compartments where increases occur include those important for priming and functional modulation of immune cells (thymus, spleen, and peripheral lymph nodes). Since all NO-related metabolites determined can either 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.

While nitrite penetration through skin *per se* is perhaps not surprising, the speed and efficacy with which this process occurred was 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

[1] Nitrous acid (HNO_2) has a pka of 3.4 and is thus largely (>99.9%) dissociated at physiological pH.

peaking within 5-15 min, depending on the compartment under study (Fig. 1, inset(s)). Maximal concentrations of NO-related metabolites were 3-6 fold lower following dermal application compared to intraperitoneal administration of equivalent doses (Bryan *et al.*, 2005), but no effort was undertaken to directly compare rates and extent of uptake between application routes. Topical administration of nitrite-free vehicle did not significantly change basal levels (not shown). Figure 1 depicts the effects of 10 $mg \cdot kg^{-1}$ nitrite, 15 min post application. Marked increases in nitrite, nitrate, RXNO and NO-heme species were seen in most, but not all compartments. Notable exceptions were kidney (nitrite), brain (nitrate, RXNO, NO-heme), and plasma (RXNO, NO-heme) where either no change or a decrease of select NO-related metabolites occurred. Robust increases in circulating nitrite in conjunction with lack of enhancement of plasma nitrosation products suggests S-nitrosoalbumin plays only a minor role in transferring NO equivalents from skin to other organs, at least under these conditions. Therefore, the majority of metabolic conversion into other NO-related species likely occurs within blood cells and tissues.

While there is precedence for increased NO-related metabolites in blood and tissues following systemic nitrite administration (Bryan *et al.,* 2005), information about its effects on the immune system is scarce (Ustyugova *et al.*, 2002; Cape & Hurst, 2009). Lymph nodes from vehicle-treated animals revealed remarkably high nitrite/nitrate concentrations, indicative of high NO production rates under basal conditions (Table 1). Dermal nitrite application (10 $mg \cdot kg^{-1}$) 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 although intracellular nitrite and nitrate concentrations double, S-nitrosation of T-cells drops by 45-60% shortly after dermal nitrite application. While little is known about effects of changes in protein nitrosation on immune

cell activity, various functional consequences are likely considering the importance of redox-based signalling (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).

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 administered for 4 days with the 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 Fig.1**).

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

CONFLICT OF INTEREST

DW is involved in commercially developing potential therapeutic use of ammonia-oxidizing bacteria on human skin. The other authors state no conflict of interest.

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Table 1. NO-related metabolites in immune-relevant compartments 15 min post dermal application of 10 mg·kg-1 sodium nitrite (in PBS, pH 7.4) compared to effects of vehicle (PBS) alone. The compartment denoted as "lymph nodes" is a homogenate of pooled superficial cervical, anxillary, and iliac lymph nodes (ND = not determined due to limited sample availability). Data are means ± SEM from *n* individual experiments and expressed as nmoles/g wet weight (for nitrite, nitrate, and RXNO) and pmoles/g wet weight for NO-Heme products; *P<0.05, **P<0.01 vs. vehicle.

		Vehicle (n=5)	Nitrite (n=3)
Thymus	*Nitrite*	0.07±0.04	3.36±1.33*
	Nitrate	2.96±0.64	5.13±1.56
	RXNO	0.24±0.06	0.20±0.02
	NO-Heme	4.34±2.97	11.6±1.9
Spleen	*Nitrite*	<0.04	1.44±0.57*
	Nitrate	1.17±0.15	5.92±1.00**
	RXNO	0.09±0.01	0.99±0.46*
	NO-Heme	1.24±0.85	12.2±5.86*
Lymph Nodes	*Nitrite*	3.71±1.29	8.53±1.91
	Nitrate	40.78±25.21	84.25±42.29
	RXNO	0.08±0.05	0.04±0.02
	NO-Heme	ND	ND

Figure 1.

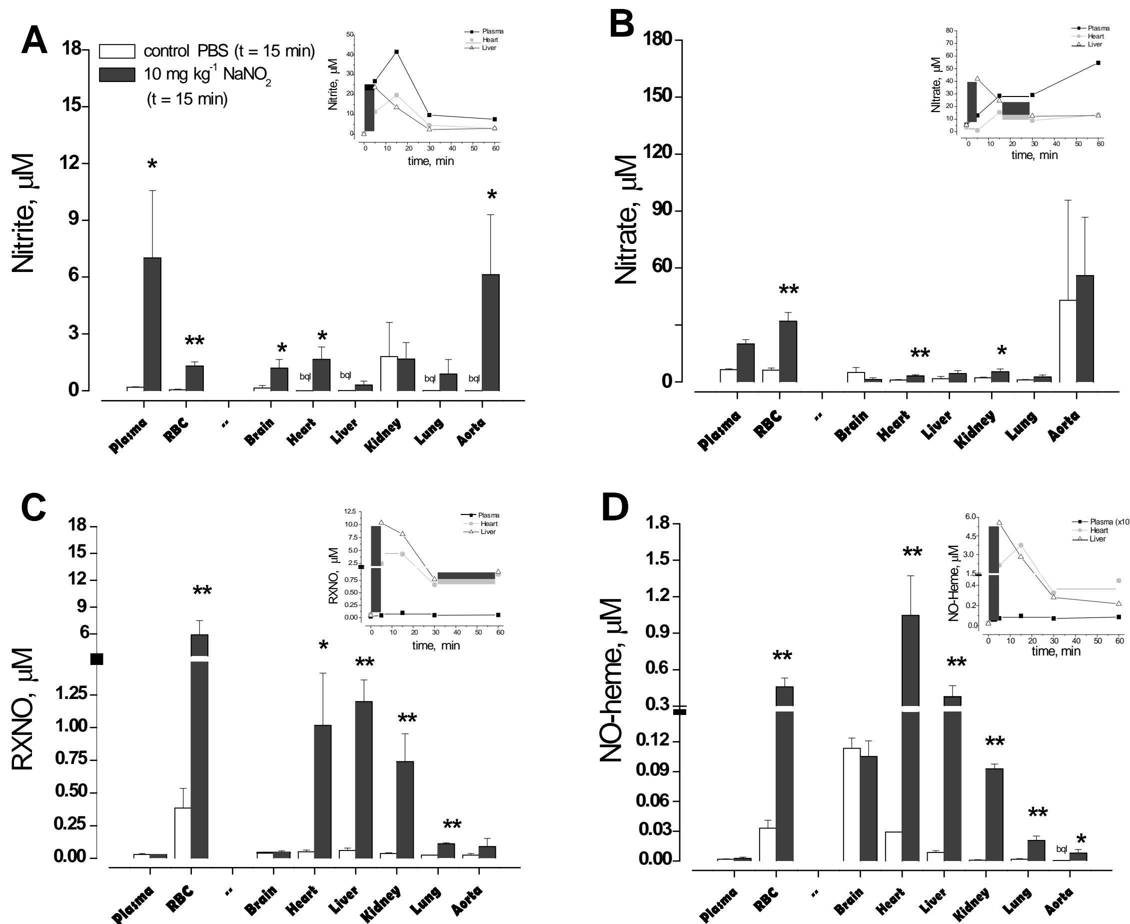


Figure 1. Concentration of NO-related metabolites in blood and tissues 15 min post-dermal application of pH-neutral solutions of sodium nitrite (10 mg˙kg-1; n=3) or vehicle (n=5) (A-D). NO-related metabolites measured include nitrite, nitrate, S- and N- nitroso (RXNO), and nitrosyl (NO-heme) species. With exception of brain, which revealed no change (RXNO, NO-heme) or decreased (nitrate) concentrations along with kidney (nitrite), appreciable increases in NO-related metabolites were seen in other compartments. Significant nitrite oxidation appears to occur only in blood, heart and kidney. Insets: Time courses of uptake and metabolism of dermal nitrite for three representative compartments (plasma, heart, liver). Within minutes marked increases in NO-related metabolites (nitrite, nitrate, RXNO, NO-heme) were evident. (RBC, red blood cell; bql, below quantifiable limit; *p<0.05, **p<0.01 vs. vehicle).

SUPPLEMENTARY INFORMATION

Materials and Methods

Experimental Animals

Male Wistar rats (250-300 g, Harlan) were kept on a regular 12h/12h light/dark cycle and allowed to acclimatize for at least 10d to local vivarium conditions prior to experimental use, with access to food (2018 rodent chow, Harlan Teklad) and regular tap water *ad libitum*. All experiments were performed at the same time of day to keep chronobiological differences to a minimum (8:30-10:30 a.m.). DO11.10 mice (Jackson Laboratory) were maintained on an ovalbumin-free diet using identical housing conditions and allowed unlimited access to food and tap water. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Dermal Application of Nitrite

Aqueous solutions of sodium nitrite ($NaNO_2$) were prepared in phosphate buffered saline (PBS; pH 7.4) immediately before use. A 2 x 4 cm section of the dorsal rat skin was gently shaved in the area just below the neck using a small cordless hair clipper 22-24h prior to nitrite application to minimize handling-related stress on the day of experimentation. Great care was taken to avoid skin irritation. Various nitrite doses were tested (0.1, 1.0, 10 mg kg^{-1} $NaNO_2$) and compared to the effect of the vehicle (PBS). Identical application volumes (800 µL) of test solutions (with nitrite concentrations adjusted according to animal weight) were applied to the shaved rat skin and evenly distributed across the defined area using the index finger (glove-covered). A time-course study (5, 15, 30, 60 min) was performed using a 10

mg kg^{-1} dose of nitrite and results were compared to application of an identical volume of PBS at 15 min which served as the basal value.

Organ Harvest and Homogenization

Heparinized (0.07 units/g body weight, i.p.) rats were anesthetized with diethylether (2 min) and euthanized by cervical dislocation. Blood was withdrawn from the inferior vena cava and immediately centrifuged at 4,600 x *g* for 8 min at room temperature (22°C) to separate red blood cells (RBC) from plasma. RBC were subjected to hypotonic lysis in water containing NEM (10 mM) and EDTA (2.5 mM). To remove blood for tissue analysis, organs were perfused *in situ* with room air-equilibrated PBS supplemented with NEM (10 mM) and EDTA (2.5 mM) for 1-2 min via the infrarenal part of the abdominal aorta, as described previously (Bryan *et al.*, 2004). Brain, heart, liver, kidney, lung, aorta, thymus, spleen, and lymph nodes were harvested sequentially, homogenized, and subjected to immediate analysis.

Lymphocytes isolation and flow cytometric/cell imaging analysis

Pooled mesenteric, axillary and cervical lymph nodes, harvested from a separate group of animals within 3-5 min after sacrifice, were immersed in ice-cold PBS, trimmed free of fat and adherent tissue, chopped into small pieces in a Petri dish, and passed through a cell strainer (BD Falcon; 100 µm). Aliquots of the resultant cell suspension (16-24 x 10^6 cells/mL) were used for protein determination, quantification of NO metabolites (after addition of NEM/EDTA and homogenization as described above), cell counting and fluorescence-activated cell sorting analysis (following a standard staining protocol using anti-CD3 antibodies from Daco). Under these conditions, 55-60% of the cells showed CD3-positive staining, with the

remainder consisting of dendritic cells, B-cells, macrophages and nodal cells as judged by microscopic examination following hematoxylin/eosin staining.

Quantification of NO oxidation, total nitrosation and nitrosylation products

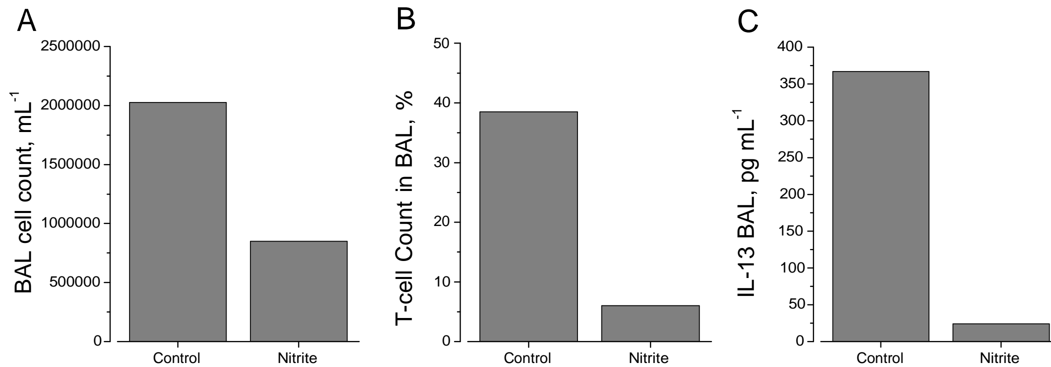
Methods for the detection of nitrosation (the sum of S- and N-nitroso species, RXNO) and nitrosyl (NO-heme) compounds, as well as the oxidation products of NO (nitrite and nitrate), in blood and tissues have been detailed previously (Feelisch *et al.*, 2002; Bryan *et al*., 2004). Quantification was achieved by group-specific denitrosation after injection of biological samples into either a triiodide-containing reaction mixture (RXNO species) or a potassium ferricyanide solution (NO-heme species) constantly purged with nitrogen, and NO was measured with the aid of a gas phase chemiluminescence detector (CLD 77am sp, EcoPhysics) under reduced ambient light (<15 lux) to prevent photolytic decomposition of NO adducts (Rodriguez *et al.*, 2003). Nitrite and nitrate concentrations were quantified by ion-chromatography (ENO20, Eicom). Protein concentrations were determined using a commercial Bradford assay (Coomassie Plus, Pierce).

Allergic Asthma Model

The effects of nitrite on T-cell specific immunestimulation was investigated in ovalbumin (OVA)-challenged DO11.10 mice. These transgenic mice harbour an OVA-specific T-cell receptor that recognizes the OVA peptide 323-339 and react to short exposures of aerosolized OVA with exacerbated airway responsiveness without requiring prior immunization. Mice used in this study were 6-7 months of age. Immediately before start of the experiment, animals were switched to a low NOx diet (TD 99396, Harlan Teklad) and divided into 3 groups (3 animal/group), a control

group receiving Milli-Q water, a low nitrite (10 mg/L $NaNO_2$ in Milli-Q water) and an intermediate nitrite group (100 mg/L $NaNO_2$ in Milli-Q water). At the day of randomization into different groups, three (control) and two (treatment groups) mice from each group were began to be exposed to a single daily challenge of aerosolized OVA (5% in PBS, 20 min exposure; Pari nebulizer) using a custom-made restraint-free inhalation chamber; one animal of either treatment group served as control for nitrite treatment alone. On the 4th day of immunostimulation and 2h after the last OVA challenge mice were euthanized using pentobarbital and lungs were flushed with 4x1 mL aliquots of cold PBS to obtain bronchial alveolar lavage (BAL) fluid. Inflammatory cell counts and T-cell-specific counts in BAL fluid were determined by light microscopy after staining with haematoxylin/eosin and anti-CD3 antibodies, respectively, using air-dried slides prepared by cytocentrifugation (Cytospin III). IL-13 concentration in BAL was determined using a commercially available ELISA (R&D Systems).

Supplementary Figure:



Supplemental Figure 1: Effects of nitrite on ovalbumin-induced airway inflammation and cytokine production in DO11.10 mice.

Nitrite (10 mg/L $NaNO_2$ in Milli-Q water) potently suppresses overall number of inflammatory cells (A), T-cell counts (B) and IL-13 production (C) in bronchiolar alveolar lavage (BAL) fluid of mice immunostimulated with aerosolized ovalbumin (5% in PBS, single 20 min exposure/day for 4 days). Mean data from two (nitrite) and three (control) animals/group, respectively. While nitrite was tolerated well in the absence of concomitant immunostimulation, ovalbumin-exposed animals on 100 mg/L nitrite died on day 1 and 2, respectively.