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Topical electrophilic nitro-fatty acids potentiate cutaneous inflammation

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Abstract

Endogenous electrophilic fatty acids mediate anti-inflammatory responses by modulating metabolic and inflammatory signal transduction and gene expression. Nitro-fatty acids and other electrophilic fatty acids may thus be useful for the prevention and treatment of immune-mediated diseases, including inflammatory skin disorders. In this regard, subcutaneous (SC) injections of nitro oleic acid (OA-NO₂), an exemplary nitro-fatty acid, inhibit skin inflammation in a model of allergic contact dermatitis (ACD). Given the nitration of unsaturated fatty acids during metabolic and inflammatory processes and the growing use of fatty acids in topical formulations, we sought to further study the effect of nitro-fatty acids on cutaneous inflammation. To accomplish this, the effect of topically applied OA-NO₂ on skin inflammation was evaluated using established murine models of contact hypersensitivity (CHS). In contrast to the effects of subcutaneously injected OA-NO₂, topical OA-NO₂ potentiated hapten-dependent inflammation inducing a sustained neutrophil-dependent inflammatory response characterized by psoriasiform histological features, increased angiogenesis, and an inflammatory infiltrate that included neutrophils, inflammatory monocytes, and $\gamma\delta$ T cells. Consistent with these results, HPLC-MS/MS analysis of skin from psoriasis patients displayed a 56% increase in nitro-conjugated linoleic acid (CLA-NO₂) levels in lesional skin compared to non-lesional skin. These results suggest that nitro-fatty acids in the skin microenvironment are products of cutaneous inflammatory responses and, in high local concentrations, may exacerbate inflammatory skin diseases.

Graphical Abstract

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Keywords

Electrophilic fatty acids; Nitro Oleic Acid; Contact hypersensitivity; Psoriasisiform dermatitis; Skin

Introduction

Reactions between nitric oxide (NO), nitrite (NO_2^-), reactive oxygen species and unsaturated fatty acids during metabolic and inflammatory processes give rise to electrophilic nitro-fatty acid nitroalkene derivatives such as nitro oleic acid (OA- NO_2) and conjugated nitro linoleic acid (CLA- NO_2). These fatty acid nitration products and their metabolites are found endogenously in plants and mammals [1]. In this regard, olives and olive oil are significant sources of fatty acids and also contain small amounts of electrophilic nitro-fatty acids, such as OA- NO_2 and LNO₂ [2]. In addition to exogenous sources, electrophilic nitro-fatty acids are mainly produced by acidic nitration reactions that occur during digestion, and are generated by oxidative inflammatory reactions, presumably as an adaptive mechanism of inflammatory resolution [3–5]. Nitro-fatty acids modulate metabolic and signal transduction reactions through post-translational modification of functionally-significant nucleophilic amino acids [6]. For example, OA- NO_2 and LNO₂ inhibit NF- κ B signal transduction by blocking p65 DNA binding, thereby suppressing the expression of

multiple pro-inflammatory cytokines and adhesion proteins [7]. Additionally, nitro-fatty acids act as partial agonists of peroxisome proliferator-activated receptor- γ (PPAR- γ) and by activating nuclear factor E2-related factor 2 (Nrf2) and the heat shock factor-regulated gene expression [8–11].

The therapeutic potential of nitro-fatty acids has been demonstrated in several murine models of inflammatory disease. In this regard, systemic administration of OA-NO₂ attenuated endotoxemia and tissue ischemia and reperfusion (I/R) injury, angiotensin II-induced hypertension, and atherogenesis [12–15]. Moreover, an allergic contact dermatitis (ACD) model demonstrated that subcutaneous (SC) injections of OA-NO₂ induced an anti-inflammatory effect that inhibited ACD by inducing a systemic immunosuppressive response [16]. On the other hand, recent studies suggest that upregulation of Nrf2, a transcription factor activated by OA-NO₂, contributes to inflammation in psoriasis by promoting keratinocyte proliferation [17]. This motivated evaluating the impact and generation of nitro-fatty acids in the regulation of cutaneous immunity. Herein we sought to directly determine the effect of topically-applied nitro-fatty acids on cutaneous inflammation. At high concentrations, topically-administered OA-NO₂ potentiated hapten-induced neutrophil-dependent inflammation, provoking a sustained psoriasis-like inflammatory response characterized by psoriasiform histological features, increased angiogenesis, and an inflammatory infiltrate that included neutrophils, inflammatory monocytes, and $\gamma\delta$ T cells.

Material and Methods

Mice and Human Tissues

Female Balb/c mice were purchased from The Jackson Laboratories (Bar Harbor, Maine) and used between the ages of 6 and 12 weeks. Mice were housed under specific-pathogen-free conditions and treated according to the University of Pittsburgh's institutional animal care guidelines and according to the NIH guide for the care and use of laboratory animals. Lesional and non-lesional skin biopsies from psoriasis patients were obtained with informed consent through the UPMC Dermatology clinic. All human samples were procured in accordance with the Declaration of Helsinki protocols and University of Pittsburgh Institutional Review Board approval.

Chemicals and Reagents

10-OA-NO₂, NO₂-¹³C₁₈OA, NO₂-CLA and ¹⁵NO₂-CLA were synthesized and purified as previously described [18–20]. The concentration of 10-OA-NO₂ and CLA-NO₂ was calculated gravimetrically or spectrophotometrically using the following extinction coefficients in phosphate buffer, OA-NO₂ ϵ_{268} 8.22 M⁻¹cm⁻¹ and CLA-NO₂ ϵ_{312} 11.20 M⁻¹cm⁻¹ [19, 21]. Solvents used for extractions and mass spectrometric analyses were of HPLC grade or higher from Burdick and Jackson (Muskegon, MI).

Nitro-fatty acid treatment and quantification

Six hundred forty, 64, and 6.4 nmoles, corresponding to 1.0, 0.1 and 0.01% OA-NO₂ solutions respectively were dissolved in either ethanol (EtOH) or dimethyl sulfoxide

(DMSO), and 20 μ l painted onto the dorsal surface of the mouse ear. Twenty-four hours following application, mouse ears were subjected to three rounds of tape stripping to remove the stratum corneum. Ears were then excised and homogenized in 20 mM phosphate buffer, pH 7.2, (2 ml) using a tissue Tearor, isotopically-labeled internal standard (0.5 ng NO₂-[¹³C₁₈]OA) was added, and sample was extracted using Bligh and Dyer method as previously reported for nitroalkene fatty acid extractions [19, 22]. The organic phase was dried under vacuum and resuspended in 500 μ l of methanol for HPLC-MS/MS analysis.

In a separate set of experiments the indicated dose of OA-NO₂ in DMSO was applied to the dorsal surface of the mouse ear then 3 and 18 h following application whole blood was collected by cardiac puncture. Subsequently, serum was obtained by 10 min centrifugation in a Microtainer tube with serum separator (Becton Dickinson; Franklin Lakes, NJ). Serum proteins (20 μ l) were subsequently precipitated using 4 volumes of cold acetonitrile in the presence of 0.1 ng NO₂-[¹³C₁₈]OA and supernatant subjected to HPLC-MS/MS analysis.

Mouse skin lipid extracts were resolved for quantitation purposes using a reverse phase HPLC column [2 $\times$ 20 mm C₁₈ Mercury column (Phenomenex; Torrance, CA)] with a gradient solvent system consisting of solvents (A): H₂O containing 0.1% acetic acid and (B): acetonitrile containing 0.1% acetic acid, at a 0.75 ml/min flow rate. Samples were applied to the column at 40% B (0.25min) and eluted with a linear increase in solvent B (40%–100% B in 2.5 min). For HPLC-MS/MS standard curves were generated using synthetic 10-OA-NO₂ and isotopically labeled standard (NO₂-[¹³C₁₈]OA and analyte quantification performed in multiple reaction monitoring mode using an AB5000 (Applied Biosystems; San Jose, CA) triple quadrupole mass spectrometer equipped with electrospray ionization sources [23, 24].

Human skin samples (6mm punch biopsy) were homogenized in phosphate buffer 20 Mm, pH 7.4 using a tissue tearor. The homogenate was spiked with 10-[¹⁵N]O₂-d₄OA (10 pmol final concentration, as internal standard) and supplemented with 20 mM HgCl₂ for 30 min at 37°C before lipid extraction using hexane:isopropanol:1M formic acid (2:1:0.1, v/v/v). The upper phase (organic phase) was dried under N₂, samples dissolved in H₂O: methanol (90:10) and loaded onto a solid phase chromatography cartridge (Thermo Scientific). Column were washed at initial conditions, dried under vacuum and eluted using methanol. Samples were dried under a stream of N₂, resuspended in 150 μ l of methanol and used for HPLC-MS/MS analysis. CLA-NO₂ was analyzed by HPLC-ESI-MS/MS using gradient solvent systems consisting of water containing 0.1% acetic acid (solvent A) and acetonitrile containing 0.1% acetic acid (solvent B) and resolved for quantitation using a reverse phase HPLC column (2 $\times$ 100 mm C₁₈ mercury column; Phenomenex) at a 0.7 ml/min flow rate. Samples were applied to the column at 30% B (0.3 min) and eluted with a linear increase in solvent B (30–100% solvent B in 8.7 min).

CLA-NO₂ was quantified following the 324.2/46 and 331.2/47 transitions (for CLA-NO₂ and 10-[¹⁵N]O₂-d₄OA respectively) using a 6500 Qtrap triple quadrupole mass spectrometer (Sciex; San Jose, CA) equipped with an electrospray ionization source. The following parameters for the mass spectrometer were used: IonSpray voltage –4.5 kV, declustering potential –80 eV, CE -42 EP -10, CXP -12, gas1 60 and gas2 45 and the source temperature 650 °C. High resolution mass spectrometric determination were performed on a Q Exactive

(Thermo Scientific). The following parameters were used in the negative ion mode: IonSpray voltage 4.0 kV, capillary temperature 325 °C, sheath gas 40, auxiliary gas 10, probe heater temperature 300 °C, S-lens 50.

Contact hypersensitivity (CHS) response

Balb/c mice were treated topically on the shaved abdomen with 20 μ L of 0.1% OA-NO₂, unless otherwise indicated, in DMSO 18 h prior to sensitization with 0.5% DNFB in acetone and olive oil, 4:1 (v:v) (Sigma-Aldrich; St. Louis, MO) on the shaved abdomen. Elicitation of the CHS response was performed 5 d later by applying OA-NO₂ 18 h prior to DNFB on dorsal surface of the right ear. In similar studies FITC or oxazolone replaced DNFB. In some studies, Ly6G Ab (4 mg/kg) was injected intraperitoneally (ip) on day -1 and day 1 of elicitation to deplete neutrophils. Measurements of ear thickness were performed at indicated times using an electronic caliper (Mitutoyo, Aurora, IL). CHS responses were assessed as an increase in ear thickness, and data is expressed as the percentage of ear thickness increase using the formula: $[(\text{thickness of challenged ear} - \text{thickness of control ear}) / (\text{thickness of control ear}) \times 100]$. For histological and real-time quantitative RT-PCR (qRT-PCR) analysis of these samples, mice were euthanized at various time points following elicitation and samples were obtained from ear skin and embedded in OCT or placed in RNAlater solution (Ambion; Austin, TX) until ready to process.

Cutaneous microscopy

To assess the histology and to characterize the inflammatory infiltrate, cross-sections of mouse ears were prepared and stained as previously described [25]. Briefly, cross-sections were embedded in Tissue-Tek OCT (Miles Laboratories; Elkhart, IN), snap frozen in pre-chilled methyl-butane (Sigma-Aldrich), and stored at -80°C until ready to use. Cryostat sections (8 μ m) were mounted onto slides pre-treated with Vectabond (Vector Laboratories; Burlingame, CA), air-dried, and fixed in cold 96% EtOH (10 min) and used for H&E staining or immunofluorescence labeling. For immunofluorescence, tissue sections were blocked with 10% normal goat or donkey serum in PBS and the avidin/biotin blocking kit (Vector Laboratories) if necessary, and immunofluorescently labeled with combinations of Abs against CD11b: Alexa 488, Gr-1: Alexa647, CD3: Alexa488, CD31: Alexa647, F4/80: Biotin, and γ δ TCR: Biotin (all from BioLegend), MHC-II: Biotin (eBioscience; San Diego, CA), and LY6C: Alexa647 (AbD Serotec; Raleigh, NC). In some experiments, OA-NO₂ was biotinylated as previously described [26] and 0.01% OA-NO₂-Biotin was topically applied to detect OA-NO₂'s ability to penetrate the skin. Biotinylated OA-NO₂ was followed by either Cy2: streptavidin or Cy3: streptavidin (JacksonImmuno; West Grove, PA). Nuclei were counter-stained with 4'6-diamidino-2-phenylindole 2HCl (DAPI; Molecular Probes; Eugene, OR). Images were acquired using an Olympus Provis AX-70 microscope system (Olympus) with FluoView 500 software. In some experiments, cells were counted during microscopic examination in 10–15 40 $\times$ high powered fields.

Real-time qRT-PCR

Real-time qRT-PCR experiments were conducted using total RNA extracted from full thickness ear skin utilizing TRI-reagent (Molecular Research Center; Cincinnati, OH) according to manufacturer's instructions, and bromochloropropane for the extraction step.

RNA concentrations were quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.; Waltham, MA). For RT analyses, RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen; Hilden, Germany) according to manufacturer's instructions. All cDNA samples were amplified with IDT PrimeTime qPCR assays (IDT; Coralville, IA) specific for B2M (endogenous control), IL-1 β , IL-6, IL-17a, IL-23a, Hmox1, NOS2, Nqo1, Ptgs2, and S100a9 and Taqman Gene Expression Assays (Life Technologies) specific for TNFS10 (TRAIL), VEGFa and 18s (endogenous control). Each PCR reaction was prepared utilizing the Taqman Gene Expression Master Mix (Life Technologies) according to manufacturer's instructions. Reactions were run on a StepOne Plus sequence detection system (Applied Biosystems) as follows: 50°C for 2 min to activate the DNA polymerase followed by 95°C for 10 min; 40 cycles were run at 95°C for 25 sec followed by 60°C for 1 min. Analysis was performed using the StepOne Plus sequence detection system software (Applied Biosystems). Relative fold-changes of mRNA expression were calculated and normalized based on the 2^{-C_t} method except for IL-23a and NOS2, which were normalized based on the 2^{-C_t} method and expressed as arbitrary units, due to expression levels being below the limit of detection in the naïve normalizing controls.

Statistical analysis

Results from multiple different groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Changes to the groups that occurred overtime were compared by a two-way ANOVA followed by a Bonferroni post-hoc test. Human samples were analyzed using a one-tail paired *t*-test. A *p* value of < 0.05 was considered statistically significant.

Results

Bioavailability and irritant effect of topical application of OA-NO₂

Previous *in vivo* studies demonstrating the beneficial health effects of electrophilic fatty acids administered OA-NO₂ systemically to attenuate endotoxemia, tissue ischemia and reperfusion (I/R) injury, angiotensin II-induced hypertension, atherosclerosis, and allergic contact dermatitis [12–16]. Therefore, OA-NO₂ was chosen as a highly representative electrophilic nitro-fatty acid (NO₂-FA) that is readily synthesized and stable. To address whether topical application of OA-NO₂ induces an anti-inflammatory response capable of suppressing CHS reactions, the capacity of OA-NO₂ to penetrate the stratum corneum of the skin was first assessed. 1% OA-NO₂ dissolved in either EtOH or DMSO was painted on the dorsal surface of the mouse ear. Mice were sacrificed 24 h later and ears were subjected to several rounds of tape stripping to remove the stratum corneum and remaining OA-NO₂ that had not penetrated the stratum corneum. The concentrations of OA-NO₂ and its major metabolites in the mouse ear were then quantified. Both solvents facilitated OA-NO₂ penetration, with DMSO acting as a more favorable solvent for OA-NO₂ penetration of the stratum corneum as determined by the mass spectrometric detection of OA-NO₂ in the lower epidermal and dermal layers of the skin (data not shown). Furthermore, in a dose response study, penetration and systemic distribution of varying concentrations of topically applied OA-NO₂ were determined. OA-NO₂ and its major metabolites were detected in the skin

across a range of topically applied concentrations (1.0%, 0.1%, 0.01%) (Figure 1A). To visualize OA-NO₂ in the skin, 0.01% biotinylated OA-NO₂ was topically applied to the dorsal surface of the mouse ear, animals sacrificed, and ears processed for image analysis 2 h post-application. Biotinylated OA-NO₂ was detected by immunofluorescence in an even distribution in the basal layer of the epidermis (Figure 1B). Together, these data indicate that OA-NO₂ penetrates and is biologically active in the skin based on the metabolites also detected there. At the highest concentration (1% OA-NO₂) physiologically-relevant levels of OA-NO₂, in the context of instigation of systemic signaling responses [27], were detected in the serum 3 h following topical application and remained detectable for at least 18 h (Figure 1C). Minimal but detectable levels of OA-NO₂ were present in serum 3 h after 0.1% and 0.01% OA-NO₂ application. Overall, these data confirm the bioavailability of topically-administered OA-NO₂ both locally in the skin and systemically at higher concentrations.

Other electrophilic compounds utilized as therapeutics, such as dimethyl fumarate, induce contact dermatitis when applied topically [28]. Thus, to determine whether OA-NO₂ alone could act as a skin irritant or induce CHS, Balb/c mice were first treated on the dorsal surface of the ear with decreasing doses of OA-NO₂ and ear thickness was assessed 24 and 48 h following topical application. 1% OA-NO₂ induced a significant increase in ear thickness compared to naïve controls at both 24 and 48 h. In contrast, doses less than 1% OA-NO₂ did not have an effect on ear thickness (Figure 1D). Subsequently, mice were sensitized with decreasing doses of OA-NO₂ on the abdomen and 5 d later were challenged on the ear with the corresponding dose of OA-NO₂. There was not an observed significant increase in ear thickness following elicitation with any of the tested concentrations (Figure 1E). Slight, but not significant increases in ear thickness were observed with 1.0% and 0.5% OA-NO₂ (Figure 1E). Vehicle control had no effect on ear thickness (data not shown). Thus, these results indicate that OA-NO₂ alone is not capable of inducing an adaptive CHS response in mice, but that concentrations of OA-NO₂ at 1% and 0.5% might have some direct irritant effect. To avoid an irritant effect by OA-NO₂ and to minimize systemic effects, 0.1% OA-NO₂ dissolved in DMSO was utilized for subsequent experiments.

Topical OA-NO₂ potentiates CHS responses

To directly test the impact of OA-NO₂ on a CHS response induced by DNFB, OA-NO₂ was topically applied 18 h prior to DNFB sensitization and elicitation. DNFB was applied to the same site on the abdomen (sensitization) and ear (elicitation) to which OA-NO₂ was applied 18 h prior (Figure 2A). Unexpectedly, there was a significant increase in the CHS response manifested by ear swelling in the presence of OA-NO₂ compared to DNFB treatment alone, at both 48 and 72 h following elicitation (Figure 2B). Vehicle control had no effect on the development of the CHS response (data not shown). Furthermore, the OA-NO₂-stimulated response was characterized by a significant increase in IL-17 mRNA expression, compared to DNFB treatment alone, at 12 and 24 h following elicitation of the CHS response (Figure 2C). To determine whether this immune potentiation was specific to DNFB, OA-NO₂ was similarly topically applied 18 h prior to FITC and oxazolone sensitization and elicitation. Consistent with DNFB findings, OA-NO₂ significantly increased the CHS responses induced by either FITC (Figure 2D, 48 and 72 h) or oxazolone (Figure 2E, 24 and 48 h). Overall, these results indicate that OA-NO₂ potentiates hapten-induced CHS responses.

Assessment of molecular phenotype of skin following topical OA-NO₂ application

To evaluate the molecular effects of OA-NO₂ in the cutaneous microenvironment, mouse ears were treated with topical OA-NO₂ prior to elicitation and sensitization (as in Figure 1A), and skin was evaluated for changes in gene expression 24 and 72 h post-elicitation. In line with the substantial increase in ear swelling, there was an increase in the mRNA expression levels for factors associated with severe dermatitis; IL-1 β , IL-6, iNOS, S100A9, IL-23, and TRAIL were significantly increased in the DNFB + OA-NO₂ treatment group 24 h following elicitation, compared to the DNFB alone treatment group (Figure 3). By 72 h, mRNA levels for these mediators were receding. Previous *in vitro* studies have demonstrated that OA-NO₂ induces antioxidant-response element (ARE)-regulated genes including HMOX-1 and NQO1, which are Nrf2 target genes [11]; thus, we examined topical application of OA-NO₂ to determine whether the ARE-regulated responses were induced in the skin following application of OA-NO₂. In collaboration with the enhanced pro-inflammatory cytokine response, there was a significant increase in both HMOX-1 and NQO1 24 h after elicitation in the DNFB + OA-NO₂ treatment group (Figure 3). COX-2, a source of both inflammatory and immunosuppressive mediators such as anti-inflammatory electrophilic omega-3 fatty acid derivatives [29], was significantly increased following topical OA-NO₂ treatment compared to DNFB alone, consistent with a pro-inflammatory role in this setting (Figure 3). Thus, this molecular analysis is consistent with observed effects of OA-NO₂ on functional CHS responses. Topical application of OA-NO₂ potentiates pro-inflammatory responses even in the presence of HMOX1 and NQO1.

Topical OA-NO₂ intensifies and prolongs the inflammatory response induced by DNFB

To further assess the pro-inflammatory mechanisms potentiated by topical OA-NO₂, it was investigated whether OA-NO₂ was important during the sensitization phase or the elicitation phase. For this, Balb/c mice were sensitized with DNFB or not (control) in the presence or absence of OA-NO₂ application to abdominal skin. Five days later, the CHS response was elicited in the ear with DNFB in the presence or absence of OA-NO₂ treatment (as in Figure 1A). In line with previous results, treatment with DNFB + OA-NO₂ applied during both sensitization and elicitation demonstrated significantly increased ear swelling compared to sensitization/elicitation treatment with DNFB alone (Figure 4A). Similarly, there was a significant increase in ear thickness if OA-NO₂ was applied with DNFB to the ear only during elicitation (Figure 4A). However, compared to DNFB treatments alone, no significant differences existed when OA-NO₂ was applied to the abdomen during sensitization with DNFB, but not for elicitation. Interestingly, without prior DNFB sensitization on the abdomen, DNFB + OA-NO₂ “elicitation” resulted in a significant increase in ear thickness at the later 6 d time point, compared to DNFB treatment alone (Figure 4A). Taken together, these results indicate that inclusion of OA-NO₂ at sensitization only marginally increases the inflammatory response, while inclusion at elicitation substantially and significantly increases inflammation. Further, even a one-time application of DNFB in the presence of OA-NO₂ is sufficient to induce the long-term pro-inflammatory effect observed.

Based on the remarkable increases in ear thickness even 6 days after elicitation, we sought to better understand the duration and kinetics of OA-NO₂-facilitated inflammation. As expected, in the control DNFB alone CHS group, the inflammatory response peaked 72 h

following elicitation. On the other hand, DNFB + OA-NO₂-induced inflammation continued to increase and peaked 10 d following elicitation. Remarkably, after a resolution phase, OA-NO₂ treated mice continued to demonstrate significantly increased ear thickness (>130%) that persisted for at least 20 d post-elicitation. This was in sharp contrast to DNFB treated controls in which the inflammatory response never reached the level of that observed in DNFB + OA-NO₂ treated animals, and completely resolved within 15 d of elicitation (Figure 4B).

Topical OA-NO₂ mediates psoriasiform inflammatory responses

The magnitude and composition of the OA-NO₂-induced inflammatory response were histologically assessed by image analysis of ear sections obtained 6 d following elicitation. Histological analysis demonstrated a psoriasiform dermatitis characterized by a dramatic increase in epidermal thickness (acanthosis) in mice treated with DNFB + OA-NO₂ during sensitization and elicitation (Figure 4C). As expected this was markedly increased compared to that observed in naïve animals or those treated with DNFB alone. Additionally, in DNFB + OA-NO₂-sensitized and -elicited mice thickening of the cornified layer (hyperkeratosis), the presence of microabscesses, sites of incomplete keratinocyte differentiation (parakeratosis), and an increase in inflammatory infiltrate were also observed (Figure 4C). Mice that were not sensitized but only elicited on the ear with DNFB + OA-NO₂ also demonstrated acanthosis and a comparable increase in inflammatory infiltrate (not shown).

One of the hallmarks of psoriasis is increased dermal vascularity. Likewise, in mice the cutaneous overexpression of pro-angiogenic molecules, such as VEGF, leads to the development of dermal angiogenesis and the subsequent development of a psoriasiform inflammation [30]. Since recent studies have demonstrated that OA-NO₂ promotes angiogenesis [31], the expression of the endothelial cell marker CD31 was examined in tissue cross-sections to assess angiogenesis induced by topical OA-NO₂ treatment. An increase in vascular CD31 expression was detected in DNFB and DNFB + OA-NO₂ treatment groups 72 h following elicitation, compared to naïve control mice (Figure 5A). Remarkably this was followed at day 6 by an extensive expansion of vascular CD31 expression observed only in the DNFB + OA-NO₂ treatment group (Figure 5A). Consistent with this, we found that expression of VEGF mRNA was markedly elevated in the skin of DNFB + OA-NO₂ treated mice 24 h after elicitation, compared to mice in the control group (Figure 5B). Thus, these data are consistent with OA-NO₂ induction of cutaneous angiogenesis.

Phenotypic analysis by immunofluorescent microscopy demonstrated that the inflammatory infiltrate 72 h following elicitation consisted of a LY6C⁺CD11b⁺ population, which was increased in the presence of OA-NO₂ compared to DNFB treatment alone (Figure 6A). Furthermore, at 72 h there was an increase in a GR-1⁺F4/80⁺MHC-II⁻ population in the dermis of the DNFB + OA-NO₂ treatment group that was only minimally present in the DNFB alone treatment group (Figure 6B). Moreover, these populations continued to increase and persist 6 d after elicitation in the DNFB + OA-NO₂ group (Figure 6). Additionally, an abundant increase was observed in F4/80⁺GR-1⁻MHC-II⁺ and F4/80⁺GR-1⁻MHC-II⁻ populations 6 d following elicitation in the DNFB + OA-NO₂

treatment group, which was only moderately present in the DNFB alone group. Thus, these data indicate that DNFB + OA-NO₂ resulted in a substantial increase in neutrophils (MHC-II⁻F4/80⁻GR-1⁺) 72 h following elicitation that was followed by a significant increase in inflammatory monocytes (CD11b⁺Ly6C⁺) and inflammatory macrophages (F4/80⁺GR-1⁻MHC-II⁺) that was evident 6 d after elicitation.

The analysis to this point suggested inflammation consistent with a psoriasiform dermatitis. In both mice and humans, $\gamma\delta$ T cells secreting IL-17 are increased in psoriatic skin compared to normal healthy skin [32, 33]. Therefore, DNFB + OA-NO₂ treated skin was evaluated for the presence of $\gamma\delta$ T cells. Increases in $\gamma\delta$ T cells at 72 h following elicitation was not detected. However, by day 6 a significant increase in CD3⁺ $\gamma\delta$ TCR⁺ T cells was observed in skin treated with DNFB + OA-NO₂ compared to skin treated with DNFB alone (Supplemental Figure 1).

Inflammation perpetuated by OA-NO₂ is dependent on neutrophils

Data in figure 6B demonstrates that neutrophils were one of the earliest populations that migrated into the skin following treatment with DNFB + OA-NO₂. Studies utilizing OA-induced lung injury and peritoneal exudate models suggest that natural OA enhances inflammatory responses by inducing neutrophil activation and migration to sites of injury [34–36]. Therefore, to determine if the increased severity of inflammation facilitated by topical OA-NO₂ is due to natural OA's propensity to attract neutrophils, we depleted neutrophils in our model of ACD. In DNFB + OA-NO₂ treated groups, neutrophil depletion with the addition of depleting Ly6G Ab significantly reduced the inflammatory response (Figure 7). In the absence of OA-NO₂, Ly6G Ab did not have an effect on the development of the CHS response (Figure 7). Thus, it appears that OA-NO₂ potentiates the inflammatory response, in part, by inducing neutrophil activation and migration.

Nitro-fatty acids are detected in psoriatic lesions

Finally, we wanted to determine if nitro-fatty acid species were present in human psoriatic lesions. Therefore, non-lesional and lesional biopsies from psoriasis patients were analyzed by HPLC-MS/MS for the detection and quantification of CLA-NO₂, the most prevalent nitro-fatty acid that shares similar properties to OA-NO₂ [3]. In support of our murine experiments, this study revealed a significant increase in CLA-NO₂ species in paired lesional vs. non-lesional biopsies (56%, $p=0.033$) and a significant pairing between samples (correlation coefficient 0.77, $p=0.04$, Figure 8). The main two peaks of CLA-NO₂ were evaluated using high resolution mass spectrometric determinations and m/z confirmed at the 1 ppm level. Fragmentation of the first peak resulted in fragments with m/z 157.0854 (<10 ppm, C₈H₁₃O₃), 171.1009 (<10 ppm, C₉H₁₅O₃) and 195.1015 (<10 ppm, C₁₁H₁₅O₃) corresponding to 12-CLA-NO₂. The second peak was characterized by m/z 224.1281 (<10 ppm, C₁₂H₁₈O₃N) and 168.1098 (<10 ppm, C₉H₁₄O₂N) corresponding to 9-CLA-NO₂. Both peaks presented the common neutral loss of 47 corresponding to HNO₂ and leading to the detection of a fragment ion with m/z 277.2163 (2 ppm). These fragmentations are in agreement with previously reported fragmentation analysis of CLA-NO₂ isomers [3]. Together these results suggest that electrophilic fatty acids may be formed at the site of inflammation and have a potentiating role in psoriasis pathogenesis.

Overall, these results demonstrate that topical application of OA-NO₂, in the presence of additional inflammatory stimuli, facilitates a severe and chronic inflammatory response that shares characteristics of psoriasis with regard to histology, IL-17 expression, increased angiogenesis, and the inflammatory infiltrate, which includes neutrophils, inflammatory monocytes and macrophages, and $\gamma\delta$ T cells.

Discussion

Systemic administration of electrophilic fatty acids mediates anti-inflammatory and metabolic signaling actions that in murine models lead to suppression of atherosclerosis, renal I/R, vascular disease, diabetes, ACD, and endotoxemia [12–16, 37]. Mechanistically, nitro-fatty acids mediate post-translational protein modification reactions that inhibit NF- κ B-regulated gene expression, induce the activation of Nrf2 and the heat shock factor-regulated transcription, and act as partial agonists of PPAR- γ [7–11]. In contrast to prior studies utilizing systemic application, topical OA-NO₂ potentiated the ACD inflammatory response induced by haptens. Importantly and consistent with observations from the topical application of other fatty acids, OA-NO₂ did not induce an inflammatory response on its own [30]. Thus, when applied to sites of inflammation, these studies support a non-specific pro-inflammatory effect mediated by topical OA-NO₂.

Murine CHS was chosen as a model of an inflammatory skin disease. A typical CHS response requires a sensitization phase that occurs following primary contact with a hapten that initiates antigen-specific T cell priming. This is followed by an elicitation phase that ensues on subsequent contact with the same hapten and results in the activation of primed T cells, recruitment of inflammatory cells, and an immense amplification of the immune response which manifests as severe ear swelling. By 72–96 h following elicitation, CHS responses typically begin to resolve. In sharp contrast, we found that sensitization and elicitation in the presence of OA-NO₂ resulted in a potent inflammatory response that was sustained for the duration of the study (20 d). The prolonged inflammation is reminiscent of a human psoriatic lesion and distinct from other inducible murine models of psoriasis, such as those elicited by topical Aldara or IL-23 injections, in which the psoriatic lesion resolves following discontinuation of treatment. Furthermore, a single application of topical DNFB + OA-NO₂ also induced significant ear swelling by day 6. In this regard, it is tempting to speculate that OA-NO₂ could induce a depot effect by retaining DNFB in the skin thereby perpetuating both sensitization and elicitation phases. However, the OA-NO₂ effect seems distinct from that described in a recently reported subchronic model of CHS that was induced by repeated application of DNFB [38]. While several hallmarks of psoriasis including epidermal thickening, hypervascularization, and increased cellular infiltration were observed, the inflammation induced by repeated application of DNFB lacked major characteristics of psoriasis including IL-17 expression and the presence of Munro's microabscesses [38]. Additionally, repeated application of haptens has the propensity to skew the immune response towards a Th2 bias [39]. Therefore, while a depot effect may play a role in the induction of the psoriasiform inflammation, the inflammatory response observed in the setting of DNFB + OA-NO₂ is distinct, and more closely resembles the full psoriatic phenotype.

Human psoriatic plaques are characterized by aberrant keratinocyte proliferation (acanthosis) and differentiation (parakeratosis), thickening of the cornified layer (hyperkeratosis), the presence of Munro's microabscesses, epidermal and dermal infiltration, increased dermal vascularization, and activation of resident myeloid dendritic cells (DCs). The inflammation induced following topical application of DNFB + OA-NO₂ was distinguished by acanthosis, hyperkeratosis, parakeratosis, increased vascularity and cellular infiltration, and the presence of Munro's microabscesses. Psoriasis is also classified as a cell-mediated autoimmune disease in which antigen-specific T helper (Th) 17, Th1, and CD8⁺ cytotoxic T cells play effector roles. In psoriatic lesions, the dermal infiltrate is further composed of $\alpha\beta$ and $\gamma\delta$ T cells, macrophages and monocytes, and neutrophils [32, 40]. Likewise, the dermal infiltrate induced by topical OA-NO₂ was characterized by an early increase in neutrophils followed by the accumulation of inflammatory monocytes, macrophages, and $\alpha\beta$ and $\gamma\delta$ T cells. Dermal $\gamma\delta$ T cells are major producers of IL-17 in mice and are required for the development of psoriasiform lesions [32]. Furthermore, the expression of several distinguishing inflammatory markers of psoriasis, including IL-1 β , IL-6, iNOS, S100A9, TRAIL, IL-23, and IL-17 was significantly increased by topical application of OA-NO₂. Together, these data indicate that the presence of OA-NO₂ during the development of CHS responses induces inflammation in the skin suggestive of psoriatic lesions.

Studies of OA-induced lung injury and peritoneal exudate models suggest that natural OA enhances inflammatory responses by inducing neutrophil activation and migration to sites of injury [34–36]. Notably, neutrophils have been associated with the pathophysiology of psoriasis and studies by Hu et al. [41] have demonstrated that an increase in neutrophil extracellular traps (NETs) and NETosis are positively correlated with disease severity. In our studies, neutrophils were one of the earliest populations that migrated into the skin following treatment with DNFB + OA-NO₂. Moreover, blockade of neutrophils inhibited the inflammatory amplification facilitated by OA-NO₂. Thus, similar to natural OA, these studies indicate that a potential mechanism of OA-NO₂ action includes the attraction of activated neutrophils to sites of inflammation, potentially by stimulating the increased expression of CD11b on neutrophils [42]. However, the molecular mechanisms that link OA-NO₂ and neutrophil activation and migration are not known. As discussed previously, OA-NO₂ administered subcutaneously leads to an anti-inflammatory effect [16] but when applied topically OA-NO₂ potentiates inflammation. We hypothesize that the difference may be due to OA-NO₂ effects on keratinocytes, which could occur in the setting of topical delivery but would be less likely following injections. Keratinocytes are a first line in host defense and source of inflammatory mediators, such as CXCL1 and CXCL20 chemokines along with IL-1 β and IL-6 cytokines that are capable of attracting and activating neutrophils, respectively. In further support of this hypothesis, studies by Sanford et al. [43] determined that when short-chain fatty acids (SCFAs) are administered topically they lead to an inflammatory response with increased IL-1 β , IL-6, and CXCL1 transcripts. Whereas when SCFAs are injected intradermally these inflammatory genes were not induced. Interestingly, the proinflammatory effects of SCFAs were mediated by inhibition of keratinocyte histone deacetylase (HDAC) activity [43]. In our model HDAC activity was not affected (data not shown) but likely a similar keratinocyte mechanism underlies this dichotomy. In this regard,

Nrf2, a prototypical pathway induced by electrophilic fatty acids, is beneficial under physiological conditions; however, sustained Nrf2 expression can lead to keratinocyte hyperkeratosis [44], a prominent psoriasis characteristic.

Topical OA-NO₂ potentiation of CHS upregulates HMOX-1 and VEGF-A, likely promoting angiogenesis. Overexpression of VEGF-A has been associated with the development of psoriasiform lesions in multiple mouse models. Heterozygous transgenic mice that overexpress VEGF-A are unable to resolve a delayed-type hypersensitivity response leading to psoriasiform inflammation and lesion development [45]. In the homozygous K14-VEGF transgenic model, mice exhibit a significant increase in angiogenesis and spontaneously develop psoriatic-like lesions at 6 months of age [30]. Further, HMOX-1 and hypoxia inducible factor-1alpha (HIF-1α), transcription factors involved in regulating angiogenesis, are significantly increased in psoriatic lesions [46, 47]. *In vitro* and *in vivo* studies from multiple laboratories demonstrate that OA-NO₂ can induce the expression of both HMOX-1 and HIF-1α, which leads to the over expression of VEGF and the induction of angiogenesis [11, 31, 48, 49]. While most properties of OA-NO₂ have been described as anti-inflammatory, Cui et al. have demonstrated that HMOX-1 is not essential for the inhibitory actions of OA-NO₂ on inflammatory cytokine secretion [7]. Thus, the increase in HMOX-1 following topical application of OA-NO₂ was not unexpected and may make an important contribution to the angiogenesis that likely contributes to the perpetuation of the psoriatic phenotype.

Aldara, a topical cream that induces psoriasiform lesions in humans and mice, has two active components; the TLR7 agonist imiquimod (IMQ) and isostearic acid, a derivative of OA [50]. Both TLR-dependent and -independent factors are required for the development of psoriasis-like lesions following Aldara application [50]. For instance, TLR7⁺ plasmacytoid DCs are activated and recruited to the skin by IMQ, while inflammasome activation, keratinocyte death, and IL-1 secretion are independent of IMQ and likely dependent on isostearic acid [50]. Interestingly, inflammasome activation and IL-1 secretion are essential for the activation and migration of neutrophils, which are necessary for the severity of inflammation observed when OA-NO₂ is present during the development of CHS. Moreover, CHS responses are dependent on TLR activation, likely through endogenous TLR ligands, such as hyaluronic acid and heat shock proteins [51]. Thus, it is possible that DNFB + OA-NO₂ stimulates a psoriasiform dermatitis through mechanisms comparable to those associated with IMQ + isostearic acid.

Electrophilic nitro fatty acid species were increased in lesional compared to non-lesional psoriatic skin. While the formation of nitrated fatty acid has been reported in mice during inflammation, this is the first report showing an inflammation-related increase in nitrated fatty acids in humans [52]. Previous modulation of CLA-NO₂ in human plasma and urine were related to increased gastric formation [53]. In addition to nitrated fatty acids, other electrophilic fatty acids are endogenously generated during inflammation via multiple oxidative reaction mechanisms and presumably contribute to the resolution of inflammation. These lipids are also capable of activating the cytoprotective Keap1-Nrf2 pathway and the heat shock response while inhibiting NF-κB activation [29, 54, 55]. Moreover, consistent with our findings, 15d-PGJ₂, an oxidized electrophilic fatty acid, can lead to the increased

expression of VEGF [56], which contributes to angiogenesis and potentially to the increase in inflammatory infiltrates. It is thus tempting to speculate that oxidized fatty acids would also potentiate inflammation. However, studies examining the effects of electrophilic fatty acids applied topically to the skin are very sparse. One such study is contrary to our findings, Coutinho et al. [57] demonstrated that when 15d-PGJ₂ was applied 'topically' to the nasal mucosa in a model of lung inflammation the inflammatory infiltrate, including neutrophils, was significantly decreased. However, Martinez et al. [58] have revealed that 15d-PGJ₂ can have a biphasic effect inducing both inflammation and resolution, depending on the microenvironment. Thus, further studies are needed to determine if other electrophilic fatty acid species will induce an inflammatory response when administered topically to sites of cutaneous inflammation. As for patients with a genetic predisposition, it appears that electrophilic fatty acids that are generated in the cutaneous inflammatory microenvironment may have a role in psoriasis pathogenesis.

Overall, these data indicate that topical administration of electrophilic fatty acids can promote and potentiate cutaneous inflammatory responses. Mechanistic differences between presumably anti-inflammatory electrophilic fatty acids generated endogenously during skin inflammation, and the pro-inflammatory effect of single-dose topically administered OA-NO₂, highlight the complexity of the cutaneous microenvironment and the balance of regulatory mechanisms that occur during active inflammation. While the activation of neutrophils and the modulation of pro-angiogenic signaling are likely important factors in the sustained psoriasiform dermatitis induced by topical OA-NO₂, these studies only begin to elucidate the mechanisms by which electrophilic fatty acids alter the immunoregulatory balance in the skin microenvironment. A better understanding of the immunoregulatory effects of nitro-fatty acids in the skin microenvironment will contribute to our understanding of the pathophysiology of inflammatory skin diseases, and may have broad impact in the development of therapeutics and fatty acid containing topical formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SC	subcutaneous
OA-NO₂	nitro oleic acid
ACD	allergic contact dermatitis
CHS	contact hypersensitivity

LNO₂	nitro linoleic acid
CLA-NO₂	conjugated nitro linoleic acid
PPAR-γ	peroxisome proliferator-activated receptor-γ
Nrf2	nuclear factor E2-related factor 2
NO₂-FA	nitro-fatty acid
ARE	antioxidant-response element
DCs	dendritic cells
HDAC	histone deacetylase
HIF-1α	hypoxia inducible factor-1alpha
IMQ	imiquimod

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Highlights

- OA-NO₂ applied to sites of cutaneous inflammation potentiates that inflammation.
- The inflammatory response induced by OA-NO₂ is dependent on neutrophils.
- Human psoriatic lesions have an increase in nitrated fatty acids.

Figure 1. Bioavailability of OA-NO₂ and its metabolites following *in vivo* topical application
 Increasing doses of OA-NO₂ in DMSO were administered topically and metabolized in skin to its respective (A, left panels) β -oxidation products and (A, right panels) through enzymatic reduction to its inactive nitroalkane products, as analyzed by HPLC-ESI-MS/MS. Representative chromatogram showing the detection of OA-NO₂ and its β -oxidation products (left panels) and reduced forms (right panels) were followed as precursors of ions with m/z 46. The presence of NO₂-12:1 was not detected. The peaks identified as (a) (right panels) correspond to the natural isotopic contribution of [¹³C₂]NO₂-18:1 present in NO₂-18:1 to the NO₂-18:0 transition and does not correspond to a metabolic product of OA-

NO₂. **(B)** Biotinylated OA-NO₂ was topically applied to the dorsal surface of the mouse ear and 2 h post-application OA-NO₂: biotin was detected by immunofluorescent techniques. Dashed line indicates epidermal: dermal junction and the arrows are pointing to areas where biotinylated OA-NO₂ is detected. **(C)** Serum quantification of OA-NO₂ following increasing doses of topically applied OA-NO₂ at time points indicated as determined by HPLC-ESI-MS/MS. **(D and E)** Balb/c mice were treated **(D)** topically on the dorsal side of the ear with decreasing doses of OA-NO₂ and ear thickness was measured 24 and 48 h following sensitization or **(E)** 5 d following abdominal sensitization CHS responses were elicited on the ear with decreasing doses of OA-NO₂. Ear thickness was measured 24–72 h following elicitation. Bars represent the mean increase in ear thickness $\pm$ SEM of 3 mice per group. Representative of 2 independent experiments. * indicates a significant difference compared to naïve controls, $p < 0.05$.

Figure 2. Topical OA-NO₂ potentiates CHS responses

(A–E) Balb/c mice were sensitized on the abdomen with (B and C) DNFB, (D) FITC, or (E) Oxazolone, as indicated, following topical OA-NO₂ treatment. 5 d later mice were elicited on the ear with DNFB, FITC, or Oxazolone, following topical OA-NO₂ application (or not, control). (A, D, and E) Ear thickness was measured 24–72 h following elicitation. Bars represent the mean increase in ear thickness $\pm$ SEM of 4–8 mice per group. Data are one representative of two individual experiments. (C) IL-17a mRNA levels were determined 12 and 24 h following elicitation with DNFB. Bars represent the mean $\pm$ SD of triplicates per group with samples pooled from three mice. Fold-change was determined using the

relative qRT-PCR 2^{-C_t} method. One representative of three individual experiments. * indicates a significant difference compared to DNFB alone treated group at each time point.

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Figure 3. Immunological responses induced following topical application of OA-NO₂
 Bar graphs demonstrate the relative fold change in mRNA expression of IL-1 β , IL-6, NOS2, S100A9, TNFS10, IL-23a, HMOX-1, Nqo1, and Ptsg2 24 and 72 h following DNFB elicitation. Fold-change was determined using the relative qRT-PCR 2^{-Ct} method, except for NOS2 and IL-23, which are expressed as arbitrary units determined by 2^{-Ct} . Data expressed as mean $\pm$ SD of triplicates per group with samples pooled from three mice. Representative of two independent experiments. Asterisk indicates a significant difference compared to DNFB treatment alone for each time point, $p < 0.05$.

Figure 4. Characterization of chronic inflammation induced in the presence of OA-NO₂ CHS was induced in Balb/c mice pretreated with OA-NO₂ (or not, controls). **(A and B)** Ear thickness was measured at the indicated times post-elicitation. ON = OA-NO₂, DN = DNFB. The mean increase in ear thickness $\pm$ SEM of 4–8 individual mice is shown. Data are one representative of two individual experiments. **(A)** * indicates a significant difference between indicated groups. NS=not significant. **(B)*** indicates a significant difference compared to the non OA-NO₂ treated group at each time point. **(C)** Six days following elicitation ears were excised and cross-sections were stained with H&E. Black Bar = 50 μ m. Red bar indicates epidermal thickness and black arrow indicates hyperkeratosis. Inset is a representative site demonstrating parakeratosis and a microabscess.

Figure 5. Topical OA-NO₂ in the presence of an inflammatory stimulus induces extensive dermal angiogenesis

(A) DNFB CHS responses were initiated following treatment with topical OA-NO₂. 72 h and 6 d following elicitation ears were excised and cross-sections were immunofluorescently labeled with CD31-specific antibodies (red) in addition to DAPI nuclear counterstain (blue). Dashed line indicates epidermal-dermal junction. White bar = 50 μ M. (B) VEGF expression was assessed 24 and 72 h following DNFB elicitation. Fold-change was determined using the relative qRT-PCR 2^{-Ct} method. Data expressed as mean $\pm$ SD of triplicates per group with samples pooled from three mice. Asterisk indicates a significant difference compared to DNFB treatment alone for each time point, unless otherwise indicated, $p < 0.05$. Representative of two independent experiments.

Figure 6. Inflammatory infiltrate characterized following treatment with DNFB and OA-NO₂ CHS responses were induced in Balb/c mice with DNFB in addition to topical OA-NO₂ (or not, controls). Five days later the CHS response was elicited on the ears with DNFB in addition to OA-NO₂ pretreatment (or not, control). 72 h and 6 d following elicitation ears were excised and cross-sections were immunofluorescently labeled with combinations of antibodies specific for **(A)** LY6C (red) and CD11b (green) or **(B)** MHC-II (green), F4/80 (red) and GR-1 (magenta). DAPI nuclear counter stain (blue). White Bar = 50 μm. Dashed line indicates the epidermal-dermal junction.

Figure 7. Inflammation perpetuated by OA-NO₂ is dependent on neutrophils

CHS was induced in Balb/c mice pretreated with OA-NO₂ (or not, controls). Some groups also received ip injections of Ly6G Ab one day prior to elicitation and one day following elicitation. Ear thickness was measured 48 h post-elicitation. The bars represent the mean increase in ear thickness $\pm$ SEM of 5 mice per group. Data are one representative of three individual experiments. Asterisks indicate a significant difference between indicated groups. * = $p < 0.05$ and *** = $p < 0.001$.

Figure 8. Detection of electrophilic nitro-fatty acid species in human psoriasis lesions

A) CLA-NO₂ content in paired lesional and non-lesional skin biopsies from psoriasis patients quantified by HPLC-MS/MS. B) Representative chromatogram (sample (a)) of non-lesional, lesional CLA-NO₂ peaks and CLA-NO₂ synthetic standard. C) High resolution mass confirmation at sub ppm level. Inset shows elution profile of CLA-NO₂ obtained during high resolution mass determinations. *= $p < 0.05$ one tail paired t-test, n=6 pairs).