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Substance P Enhances Wound Closure in Nitric Oxide Synthase Knockout Mice

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Introduction. **The neuropeptide, substance P (SP), up-regulates nitric oxide production (NO). The purpose of this study was to determine whether SP enhances response to cutaneous injury in nitric oxide synthase knockout (NOS null) mice.**

Methods. **We studied mice with targeted deletions of the 3 NOS genes, neuronal NOS, inducible NOS, or endothelial NOS. Full thickness dorsal wounds were treated daily (d 0–6) with topical SP or normal saline (NaCl). Wounds were analyzed by flow cytometry for macrophage, leukocyte, endothelial, and dendritic cells. Healing time and wound epithelialization were compared using analysis of variance.**

Results. **Wound closure in the 3 NOS null mice was slower than the control mice ($P < 0.05$). SP treatment enhanced wound closure in NOS null mice ($P < 0.02$). NOS null wounds exhibited reduced inflammation. SP increased macrophage, leukocyte, and dendritic cell densities at d 3 and d 7 ($P < 0.05$) in all NOS null mice. SP increased endothelial cell number in neuronal NOS and inducible NOS null mice, but not in endothelial NOS null mice ($P > 0.05$).**

Conclusions. **SP ameliorated the impaired wound healing response observed in NOS null mice by enhancing wound closure kinetics and epithelialization. SP increased inflammatory cell density in the wounds supporting the essential role of inflammatory cells, especially macrophages, in wound repair.**

Key Words: **SP; wound healing; response to injury; neuropeptide; NOS; iNOS; eNOS; nNOS; inflammation; image analysis; flow cytometry.**

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INTRODUCTION

Substance P (SP), a proinflammatory neuropeptide released from sensory nerves, modulates inflammatory responses to cutaneous injury [1], including vascular relaxation and permeability [2–6], mediator production [7, 8], adhesion molecule expression [9–11], and migration and proliferation of inflammatory and endothelial cells [12–15]. SP has long been recognized to induce secretion of a short acting mediator known as endothelium relaxing factor [16], which has been identified to be nitric oxide (NO) [17–19], a free radical that mediates physiological and pathophysiological processes with beneficial and deleterious effects. Three distinct enzymes catalyze NO production from arginine: neuronal nitric oxide synthase (nNOS/NOS1), inducible nitric oxide synthase (iNOS/NOS2), and endothelial nitric oxide synthase (eNOS/NOS3) [6, 20–22]. SP has been shown to up-regulate enzyme activity of iNOS and eNOS [23, 24].

NO is essential for adequate wound healing [25–28], sensory neuron differentiation [29] and repair [30, 31], vascular homeostasis [16], angiogenesis [32], leukocyte activation and cytokine up-regulation [33]. All 3 NOS isoforms are expressed in skin [34–37]. NO levels are elevated in normal burn wounds [38, 39] and mice with targeted deletion of each of NOS genes display delayed cutaneous wound healing [40–42].

We have previously documented that SP improves wound healing responses in a diabetic murine excisional model of impaired cutaneous healing [43]. Whether this beneficial effect involves SP modulation of NOS activity has not been determined. The purpose of this study was to determine how NO might be involved in mediating SP induced proinflammatory events. To address this mechanism, we examined the

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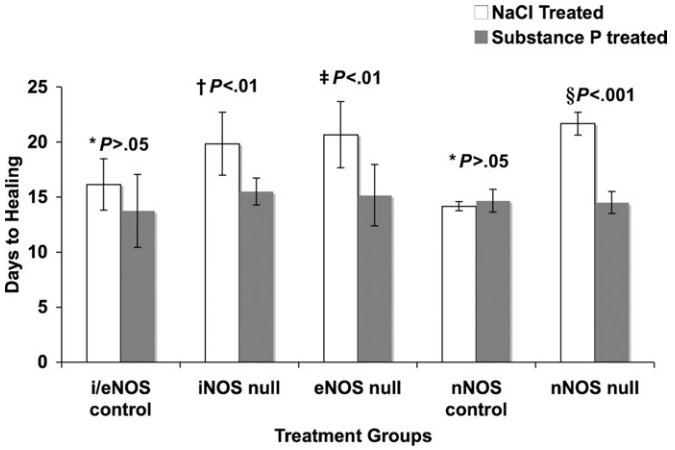

FIG. 1. SP improves wound healing kinetics in mice with targeted deletion of each of the NOS isoforms. NOS knockout mice treated with topical SP healed faster than those treated with NaCl (†, ‡, § $P < 0.01$). There was no significant difference in time to closure between treatment groups in the control mice ($P > 0.05$). Error bars represent standard deviation.

effect of SP on wound healing kinetics in mice with targeted deletion of each of the 3 NOS isoforms and in their respective background controls. We hypothesized that SP would not improve the wound healing response in the NOS null mice.

METHODS

Animal Model

Animal experiments were conducted with 5 groups of mice (8 to 10 wk old) purchased from the Jackson Laboratory (Bar Harbor, ME): (1) iNOS null, (2) eNOS null, (3) nNOS null, (4) iNOS/eNOS controls (C57BL/6J), (5) nNOS controls (B6129SF/2J) according to IACAC guidelines with approval from the University of Washington Animal Care Committee.

Time to Closure Experiment

Time to closure experiments were performed as described previously [44, 45]. Briefly, a full thickness dorsal 6-mm excisional wound was created on the dorsal surface with a dermal biopsy punch (Miltex Instrument Co. Inc., Bethpage, NY) and covered with a semiocclusive dressing (Tegaderm; 3M, St. Paul MN) affixed with an adhesive (Mastisol; Ferndale Laboratories, Detroit, MI) to minimize removal of the dressing. Wounds (6 mice/treatment group) were treated daily for 7 d (d 0–6) with topical application of 50 μL of either SP (10^{-7}M) or normal saline (NaCl) by infusion with a 26-gauge needle through the dressing onto the wound bed. The wounds were examined daily during the 1st wk and subsequently 5 times per wk until clinical wound closure. Wounds were photographed on d 0 and on the day of closure and harvested at the day of closure for histology.

The wounds were fixed in either zinc Tris buffer (0.1 M Tris, pH 7.4, 0.5 g/L calcium acetate, 5.0 g/L zinc acetate and 5.0 g/L zinc chloride) for immunohistochemistry [46] or 10% neutral buffered formalin for histology [47] and paraffin embedded.

Wound Analysis

Using digital images of the wounds, the wound area at d 0 and at closure was measured using Adobe Photoshop 7.0 containing the Image Processing Tool Kit plug-in (Reindeer Graphics, Ashville, NC) by 2 independent observers. As previously described [45], wound epithelialization was calculated according to the following equation:

$$\text{Wound epithelialization} = 1 - \frac{\text{Wound size at closure}}{\text{Wound size at d 0}}$$

Nitric Oxide Analysis in Skin and Wounds

NO production in the murine skin and wounds was determined by measuring the stable NO breakdown products nitrite and nitrate using a modified Griess reagent assay kit (Assay Designs, Inc., Ann Arbor, MI). Briefly, dorsal skin and wounds were harvested and homogenized at 4°C in 50 mM TRIS HCl buffer (pH 7.4; 1 mL/100 mg tissue) containing 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich, St. Louis, MO) and 1 μmM pepstatin A (Peninsula Laborato-

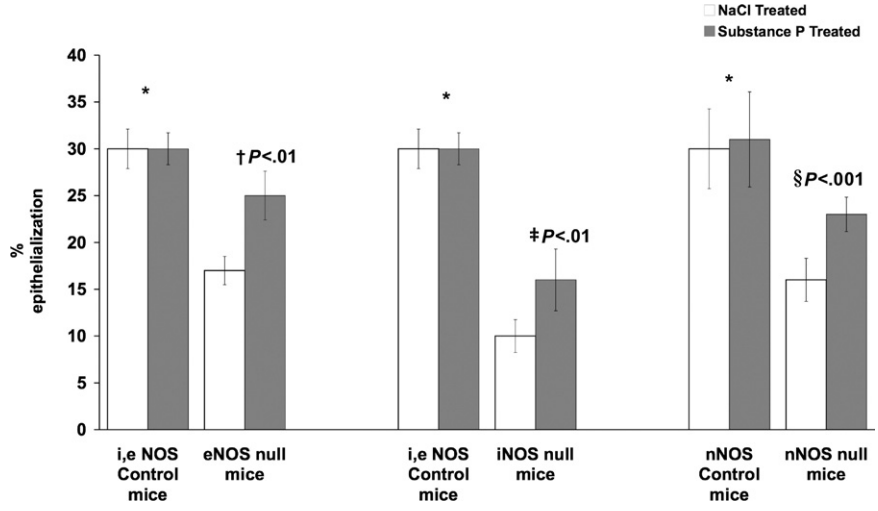

FIG. 2. SP treatment resulted in a statistically significant increased wound epithelialization compared with NaCl treatment in each of the NOS null groups (†, ‡, § $P < 0.01$). SP treatment had no effect on wound epithelialization in the control mice (*$P > 0.1$). Error bars represent standard deviation.

ries, San Carlos, CA). Homogenate (50 μL) was incubated with nitrate reductase to convert nitrate to nitrite followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction. Absorbance was measured at 540 nm and the concentration of nitrite (μmol/L) was calculated from a nitrite standard curve.

The protein concentration (g/L) in each skin and wound sample was quantified by BCA assay (Pierce, Rockford, IL) according to the manufacturer's instructions. The concentration of nitrite in each sample was standardized with the protein concentration and results were expressed as μMol nitrite/g protein.

Flow Cytometry on Wound Samples

To examine the inflammatory response in the wounds, we used tissue dispersion and flow cytometry, which has been validated as a technique for quantifying wound cellular composition [48]. Briefly, 1.5 $\times$ 1.5 cm full-thickness, excisional wounds were created on the dorsal surface of the 5 groups of mice [48]. Wound corners were tattooed to mark the original wound margins and the wounds were covered with a semiocclusive dressing as above. Mice were randomly assigned to treatment with 300 μL of daily topical infusion of either 10^{-7}M SP [43] or NaCl onto the wound beds. Wounds were harvested

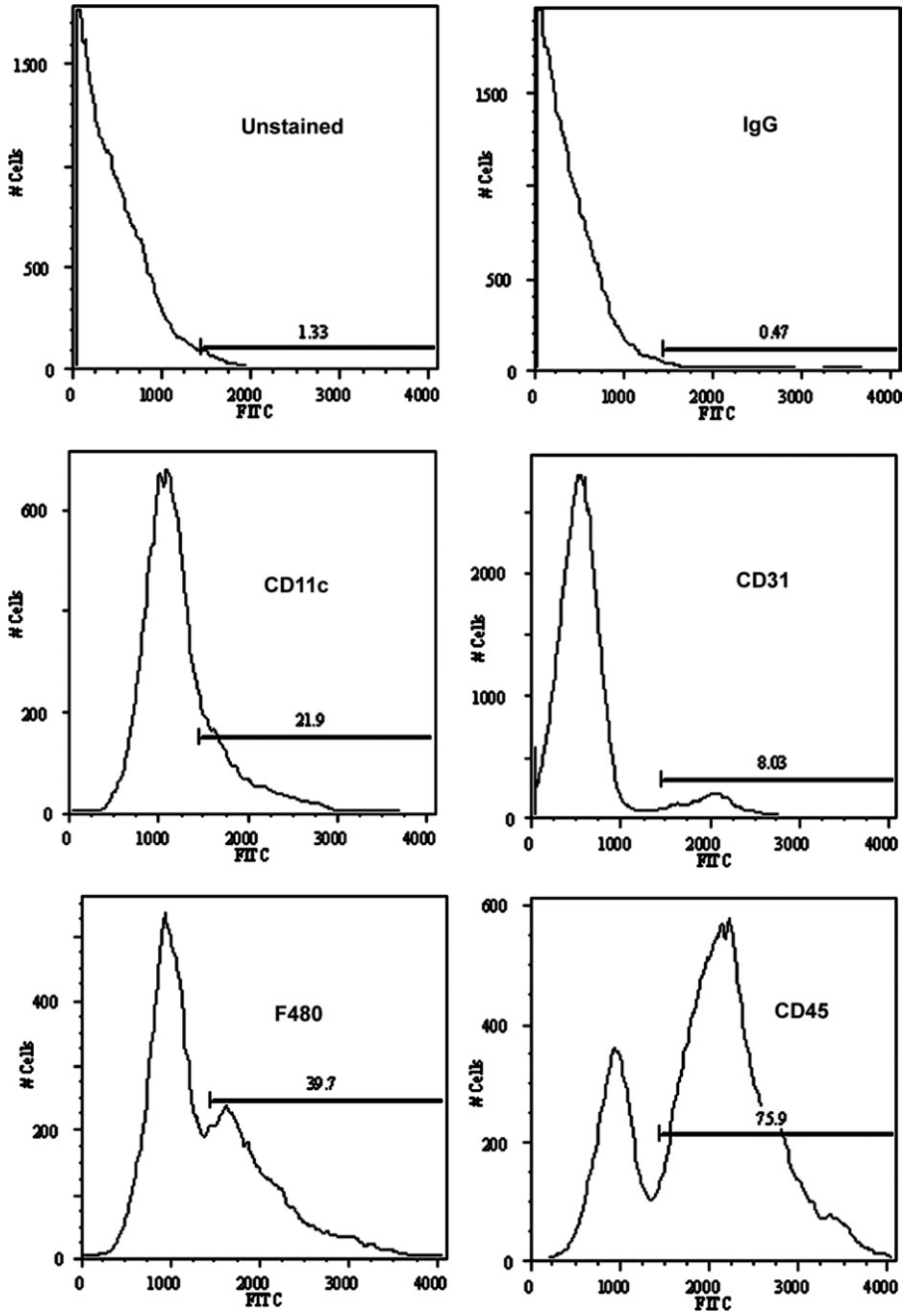

FIG. 3. Flow cytometry was performed on cells stained with fluorescein isothiocyanate-labeled antibodies (CD11c for dendritic cells; F4/80 for macrophages; CD45 for leukocytes; CD31 for endothelial cells.) For each experiment, cell gating was set between 1% and 2% of the unstained controls and that gate was applied to all conditions in the same experiment. In the data shown here, the cell gating for unstained cells was set at 1.33% of total cell population, and this gate was used for each of the other conditions.

at the tattooed margins on post-wounding d 3 and d 7 (3 mice per treatment group at each time point). Tissue dispersion and cell harvesting for flow cytometry was performed using enzymatic digestion in dispase and hyaluronidase [48]. Cell counts were performed to determine total cell density. Flow cytometry (FACScan with CellQuest Pro software; Becton Dickinson and Co., Franklin Lakes, NJ) was performed after immunostaining cells with fluorescein isothiocyanate-labeled antibodies (F4/80 for macrophages; CD45 for leukocytes; CD31 for endothelial cells; CD11c for dendritic cells and rabbit IgG for controls). For each experiment, cell gating was set between 1% to 2% of the unstained controls and that gate was applied to all conditions in the same experiment. Overall cell density was calculated using total cell counts and weights of each sample. Density of each cell type was calculated by applying percent values to overall total cell density in each sample.

Immunohistochemistry and Image Analysis

Cutaneous nerves were localized on the 6-μm sections of paraffin embedded skin samples using indirect immunoperoxidase with a polyclonal antibody against the general nerve marker, protein gene product 9.5 (PGP 9.5; Accurate Antibodies, Inc., Westbury, NY) and diaminobenzidine as the chromagen. Nonoverlapping digital images (25×) of each tissue section were taken with bright field illumination on a Nikon SA Microphot upright light microscope (www.Nikonusa.com; Meridian Instruments Company Inc, Kent WA). Digital images were imported into Adobe Photoshop 7.0 containing the Image Processing Tool Kit plug in (Reindeer Games, Inc.). Computer-assisted image analysis was used to quantify PGP 9.5 positive profiles which reflected nerves in the immunostained skin as previously described [49]. The number and area of PGP 9.5^+ profiles per dermal area (μm^2) in each skin sample was measured. In each image, the peroxidase/diaminobenzidine brown reaction product was isolated through a scripted process of background color removal as previously reported [49]. Two observers, blinded to treatment, analyzed all specimens. Interuser reliability and differential PGP 9.5+ profile counts in each group were confirmed using 1-way analysis of variance.

SP Levels in Murine Skin

Denuded dorsal skin was excised, frozen, and stored in liquid nitrogen. While frozen, the tissue was weighed, cut into 2-mm pieces, and pulverized in liquid nitrogen using a mortar and pestle. SP was extracted from skin using acid extraction [50]. Briefly, pulverized tissue was resuspended in 2 M acetic acid (5 mL/g tissue) containing 1 mM ethylenediamine tetraacetic acid, 1 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, heated at 100°C for 30 min and centrifuged for 10 min at 10,000 × g at room temperature. Extracts were assayed for SP using the correlate-EIA SP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. SP levels were normalized to sample protein concentration.

Statistical Methods

Potential differences in NO levels (μmol nitrite/g protein) or SP in uninjured skin and wound samples from the null mice and their respective controls were determined using the Student's t-test ($P < 0.05$ was considered as statistically significant). Healing time, degree of epithelialization at time of closure, and density of specific cell types from flow cytometry in each group were compared using analysis of variance test ($P < 0.05$ was considered as statistically significant). Results are expressed as mean ± standard deviation.

RESULTS

Wound Repair Kinetics

The average time for wound closure was significantly slower in NaCl treated wounds in all 3 NOS null mice (20.6 d in eNOS null, 19.8 in iNOS null, and 21.6 d in nNOS null) compared with their respective control mice (16.1d in i/eNOS control; 14.2d in nNOS controls), ($P \leq 0.05$, Fig. 1). Topical SP treatment accelerated wound closure compared with NaCl treatment in all NOS null mice groups ($P < 0.02$), but had no effect in control mice (Fig. 1). SP also improved wound epithelialization at the day of closure in all NOS null mice compared with NaCl treatment ($P < 0.02$, Fig. 2).

SP Effect on Inflammation

Inflammatory cell response on d 3 and d 7 after wounding was determined by evaluating specific cell populations using flow cytometry. Flow cytometry plots such as the one in Fig. 3 were generated to determine percentages of dendritic cells, macrophages, leukocytes, and endothelial cells. The overall cell density in the SP-treated wounds was greater than in NaCl-treated wounds on d 3 in nNOS null and iNOS null mice, and on d 7 post-wounding in all NOS null mice ($P \leq 0.05$, Fig. 4). There were no significant differences in cell density in control mice treated with SP compared with NaCl at d 3 or d 7 ($P > 0.05$, Fig. 4). Dendritic cells, macrophages, leukocytes densities were greater in the SP-treated wounds than in NaCl-treated wounds at both d 3 and d 7 post-wounding ($P \leq 0.05$) in all NOS null mice (Fig. 5A, B, and C). Whereas SP increased endothelial cell number in nNOS and iNOS murine wounds on d 3 and d 7 ($P \leq 0.05$, Fig. 5A, C), the neuropeptide had no effect on endothelial cell number in the eNOS null mice ($P > 0.05$; Fig. 5B). Dendritic cells, macrophages, leukocytes, and endothelial cell densities

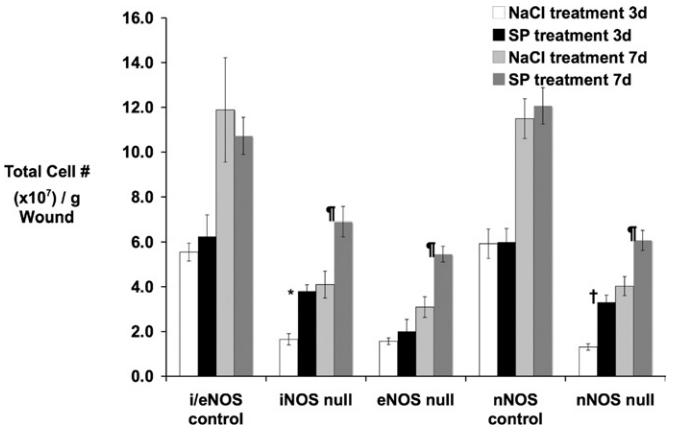

FIG. 4. Using flow cytometry, total cell density was greater in SP-treated wounds than in NaCl-treated wounds on d 3 in iNOS null (*$P < 0.01$) and nNOS null (†$P < 0.01$) mice and on d 7 post-wounding in all NOS null mice (¶$P < 0.01$). There were no significant differences in cell density in control groups treated with SP compared with NaCl at any time points ($P > 0.1$). Error bars represent standard deviation.

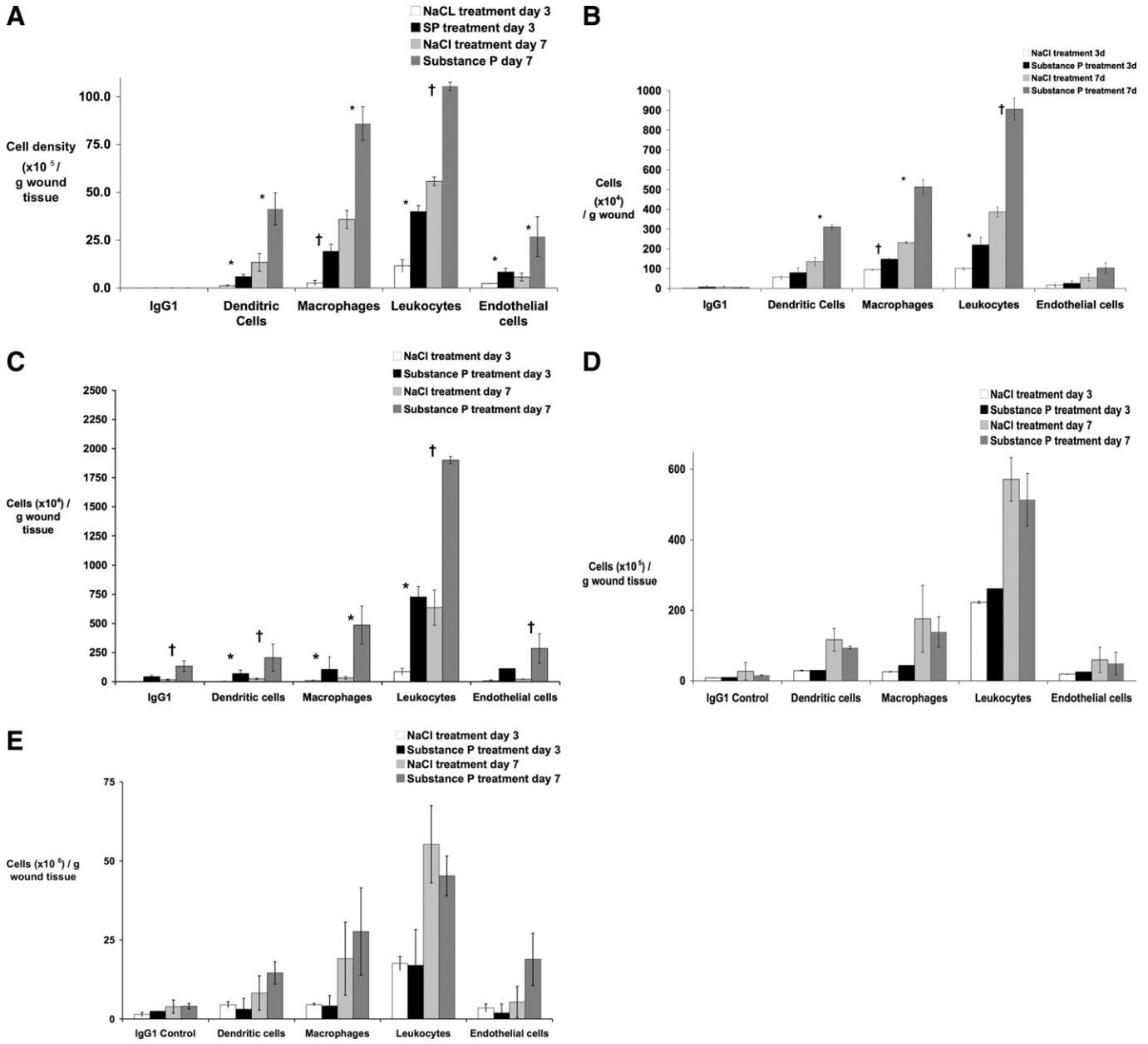

FIG. 5. In each of the NOS null mice, flow cytometry demonstrated that topical SP increased dendritic cells (CD11c), macrophages (F480), and leukocytes (CD45) in excisional wounds. This effect was not seen in the control mice. In each figure, error bars represent standard deviation. (A) In iNOS null mice, topical SP increased density of all 4 cell types on d 3 and d 7 compared with NaCl (*$P < 0.05$; †$P < 0.01$). The IgG controls had levels below detection. (B) In eNOS null mice, topical SP increased density of dendritic cells, macrophages, and leukocytes at d 3 and d 7 compared with NaCl (*$P < 0.05$; †$P < 0.01$). SP had no significant effect on endothelial cell number in the eNOS null mice at either time point. (C) In nNOS null mice, topical SP increased density of dendritic cells, macrophages, leukocytes, and endothelial cells compared with NaCl on d 3 and d 7 post-wounding (*$P < 0.01$; †$P < 0.05$). (D) In the iNOS/eNOS control mice, topical SP had no significant effect on density of dendritic cells, macrophages, leukocytes, or endothelial cells compared with NaCl on d 3 or d 7 after wounding. (E) In the nNOS control mice, topical SP had no significant effect on density of dendritic cells, macrophages, leukocytes, or endothelial cells compared with NaCl on d 3 day or d 7 post-wounding.

were not altered by SP treatment in control murine wounds at either d 3 or d 7 ($P > 0.05$; Fig. 5D, E). These results suggest that the topical application of SP enhances the local inflammatory cell response in NOS null mice but does not affect responses to injury under normal wounding conditions.

NO Measurements in Skin and Wounds

To determine whether SP altered nitric oxide levels in wounds in the NOS null mice, we measured nitrite and nitrate levels. Baseline NO levels were not significantly different (μmol/g protein) in unwounded skin of either iNOS ($P = 0.39$) or eNOS null mice ($P = 0.31$)

TABLE 1

Nitrate and Nitrite Levels in Murine Skin Wounds

	Uninjured skin	NaCl treated wounds	SP treated wounds	*P* values: NaCl *versus* SP
e,iNOS Control	15.78 ± 3.67*†	11.89 ± 6.10	12.52 ± 2.81	0.75
iNOS null	13.72 ± 2.35*	3.59 ± 1.26	9.08 ± 2.71	0.04
eNOS null	13.71 ± 4.37†	6.59 ± 0.02	15.14 ± 0.33	0.01
nNOS Control	13.52 ± 3.56‡	16.05 ± 4.98	12.18 ± 3.29	0.25
nNOS null	17.61 ± 2.09‡	7.75 ± 4.11	12.72 ± 4.79	0.89

Notes. Baseline nitrate and nitrite levels (μmol nitrate and nitrite/g protein) did not differ in uninjured skin samples from iNOS null mice (*$P = 0.39$) or eNOS null mice (†$P = 0.31$) and their controls. Nitrate and nitrite levels were slightly higher in uninjured nNOS null mice skin samples (‡$P = 0.04$) compared to controls. Topical SP increased nitrate and nitrite levels in iNOS null ($P = 0.04$) and eNOS null ($P = 0.01$) wounds compared to NaCl treatment. SP did not affect nitrate and nitrite levels in nNOS null wounds and had no effect in any control mice wounds. Data are presented as mean ± standard deviation.

*$P = 0.39$; †$P = 0.31$; ‡$P = 0.04$.

compared with their controls; unwounded skin of nNOS null mice demonstrated higher NO levels ($P = 0.04$; Table 1). Nitrate and nitrite levels in NaCl treated wounds were lower in all NOS null mice compared with their respective controls. In the SP treated wounds in null mice, nitrate and nitrite levels approached normal levels on d 7 ($P = 0.04$ in iNOS null; $P = 0.01$ in eNOS null mice; $P = 0.89$ in nNOS null mice; Table 1).

Nerve Distribution in NOS Null Mice

SP effects on wound healing responses in the NOS null mice raises the possibility that NOS null mice are deficient in SP production due to reduced nerve numbers. Using computerized image analysis [49] we confirmed that the iNOS null mice have significantly reduced number and total area of PGP 9.5^+ structures per μm^2 dermal area (Table 2). Nerve counts in nNOS skin were lower than control tissues from nNOS control mice but this did not reach significance; reduction in the area of PGP 9.5^+ profiles per dermal area in nNOS null mice approached significance. Interestingly the eNOS null mice had more nerves and increased nerve area but neither was statistically significant. These data suggest that iNOS null mice have fewer nerve fibers than control mice but that nNOS and eNOS mice have no significant reduction in cutaneous innervation.

SP Levels in NOS Null Mice

Since nerve numbers are reduced in the dermis of the iNOS skin and slightly reduced in nNOS null mice, a corresponding decrease in cutaneous SP concentration might also be expected. However, SP levels as determined by enzyme-linked immunosorbent assay were not significantly different between the NOS null mice and normal controls (Table 2). SP levels in skin from the iNOS null mice (31.8pg/mg protein) and eNOS null mice (32.5 pg/mg protein) were lower than the control mice (44.7pg/mg protein) but did not reach significance ($P = 0.06$). SP levels in the nNOS null murine skin (44.2pg/mg protein) were likewise no different than in their control mice (42.8pg/mg protein; $P > 0.05$.) It is possible that further dermal-epidermal separation would

TABLE 2

Nerve and Neuropeptide Quantification in Murine Skin

	i/eNOS control murine dermis	iNOS null murine dermis	eNOS null murine dermis	nNOS control murine dermis	nNOS null murine dermis
# PGP^+ structures/μm^2 dermis	0.0012 ± .0003	0.0007 ± .0003 $P = 0.0007$	0.0014 ± .0006 $P = 0.28$	0.0011 ± .0002	0.0007 ± .0005 $P = 0.12$
Area of PGP^+ structures/μm^2 dermis	78.3 ± 39.9	52.5 ± 39.9 $P = 0.03$	94.4 ± 45.8 $P = 0.43$	2.6 ± 21.4	53.6 ± 47.0 $P = 0.07$
SP levels (pg/mg protein)	44.7 ± 10.3	31.8 ± 3.4 $P = 0.06$	32.5 ± 6.1 $P = 0.06$	42.8 ± 17.0	44.2 ± 11.6 $P = 0.9$

Notes. The density of PGP^+ nerves (# of PGP^+ structures/dermal area) was reduced in iNOS null murine dermis ($P < 0.001$); it was increased but not significant in eNOS mice ($P = 0.28$); and low but not significant in nNOS mice ($P = 0.12$). The area fraction of nerves (area of PGP^+ structures/dermal area) was significantly reduced in iNOS null murine dermis ($P = 0.03$) and approached significance in nNOS null dermis ($P = 0.07$). No difference in nerve area fraction was observed in eNOS null dermis ($P = 0.43$). SP levels in skin from the NOS null mice were not statistically different from levels in the respective controls. Data are presented as mean ± standard deviation.

identify statistically significant differences in either the epidermis or the dermis. However, very low total cutaneous SP levels in whole skin samples suggest that assaying the epidermis and dermis separately is not logistically practical.

DISCUSSION

The skin, including the epidermis and dermis, contains nonmyelinated sensory nerve fibers [3, 51]. Sensory nerve release of tachykinins such as SP mediates proinflammatory responses [51, 52]. Nitric oxide induces many of the same downstream proinflammatory effects seen with SP. Insufficient levels of both appear to be implicated in impaired wound repair [28, 43]. In this study, we have demonstrated that topical application of SP improves wound healing kinetics in a model of impaired murine wound healing in NOS null mice and support our hypothesis that SP contributes to the dermal wound repair process.

SP activates both iNOS (calcium independent) [24], and the constitutively expressed eNOS (calcium dependent) enzymes [23]. Therefore, NO may be a key intermediary metabolite for SP-mediated responses during wound repair. As part of our attempt to understand the mechanism by which SP mediates responses to injury, we have shown that SP improves wound closure kinetics, epithelialization, and inflammatory cellular responses in the NOS null mice. In all 3 knock out models, SP enhanced numbers of inflammatory cells on d 3 and d 7 after wounding. Past studies have determined that bone marrow derived cells, specifically macrophages [53] may be crucial to normal healing responses.

Topical SP increased endothelial cell number in nNOS null and iNOS null mice on d 3 and d 7 compared with NaCl treated mice. Interestingly, SP did not increase endothelial cell number in the eNOS null mice ($P > 0.05$), suggesting that SP activation of eNOS in endothelial cells may contribute to wound repair angiogenesis. Reports of eNOS mediated endothelial cell proliferation and migration in response to vascular endothelial growth factor support our observation [54]. Despite this lack of effect on endothelial cells in eNOS null mice, SP-treated wounds healed more rapidly than NaCl treated wounds. Whereas, wound angiogenesis has been considered mandatory for wound healing, normal healing has been reported with angio-inhibition [55]. This suggests that impaired angiogenesis is not a limiting factor in healing in eNOS null mice wounds and that SP is impacting other responses to injury in these mice.

Interestingly, topical SP enhanced NO production in wounds of mice with targeted deletions of each of the 3 isoforms of the NOS genes. This suggests that SP increased activity of one of the other NOS isoforms in the null mice. Subsequent studies in our laboratory demonstrate that SP has no effect on wound healing kinetics in C56Bl6 murine wounds treated with topical N-nitro-L-arginine-methyl-ester, a nonspecific NOS inhibitor (data not shown.) This confirms the essential role that NO may have in mediating SP proinflammatory effects.

With our observation that the neuropeptide, SP, improves healing in NOS null mice and the recognized neurotrophic effects by NO on nerve growth in uninjured skin [56, 57], we evaluated nerve distribution in samples of uninjured skin from each of the NOS null mice genotypes. Whereas nerve numbers in the NOS null mice were reduced compared with control mice, this finding was only significant in the iNOS null mice. Likewise, SP levels were reduced in uninjured skin in the NOS null mice, but did not reach statistical significance. Given that in all 3 models SP improved wound healing kinetics and increased inflammatory response, a small but relative reduction in this proinflammatory neuropeptide may be sufficient to alter responses to injury.

CONCLUSIONS

SP ameliorated the impaired wound healing response observed in NOS null mice by enhancing wound closure kinetics and wound epithelialization in all NOS (eNOS, iNOS, and nNOS) null mice. Specifically, SP increased inflammatory cell density in the wounds, supporting the essential role of inflammatory cells, especially macrophages, in wound repair. Absence of SP effects on endothelial cell number in eNOS null wounds suggest that NO may mediate SP-induced wound angiogenesis.

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