Neutral endopeptidase inhibition in diabetic wound repair

MICHELLE L. SPENNY, MD[a]; PORNPROM MUANGMAN, MD[a]; STEPHEN R. SULLIVAN, MD[a]; NIGEL W. BUNNETT, PhD[c], JOHN C. ANSEL, MD, PhD[d]; JOHN E. OLERUD, MD[b]; NICOLE S. GIBRAN, MD[a]

In response to cutaneous injury, sensory nerves release substance P, a proinflammatory neuropeptide. Substance P stimulates mitogenesis and migration of keratinocytes, fibroblasts, and endothelial cells. Neutral endopeptidase (NEP), a cell surface metallopeptidase, degrades substance P. Chronic nonhealing wounds and skin from patients with diabetes mellitus show increased NEP localization and activity. We hypothesized that increased NEP may retard wound healing and that NEP inhibition would improve closure kinetics in an excisional murine wound model. NEP enzyme activity was measured in skin samples from mutant diabetic mice (db/db) and nondiabetic (db/–) littermates by degradation of glutaryl-ala-ala-phe-4-methoxy-2-naphthylamine. Full-thickness 6-mm dorsal excisional wounds treated with normal saline or the NEP inhibitor thiorphan (10 μM or 25 μM) for 7 days were followed until closure. Histological examination and NEP activity were evaluated in a subset of wounds. NEP activity in unwounded db/db skin (20.6 pmol MNA/hr/μg) significantly exceeded activity in db/–skin (7.9 pmol MNA/hr/μg; $p = 0.02$). In db/db mice, 25 μM thiorphan shortened time to closure (18.0 days; $p < 0.05$) compared to normal saline (23.5 days). NEP inhibition did not alter closure kinetics in db/–mice. While the inflammatory response appeared enhanced in early wounds treated with thiorphan, blinded histological scoring of healed wounds using a semiquantitative scale showed no difference in inflammation. Unwounded skin from diabetic mice shows increased NEP activity and NEP inhibition improved wound closure kinetics without affecting contraction, suggesting that its principal effect was to augment epithelialization. **(WOUND REP REG 2002;10:295–301)**

Aberrant wound healing is a major problem for patients with diabetes mellitus. The American Diabetes Association estimates the risk of amputation in diabetic patients with nonhealing foot ulcers at 14–24%.[1] Reasons for these abnormal responses are complicated and multifactorial. However, neuropathy represents a leading causal pathway for ulcer formation.[2,3] One study of patients with diabetes mellitus found a 15.5-fold increased risk of amputation in patients who lacked vibratory sense compared to patients with intact sensation.[4] A meta-analysis of neuropathic wounds found that only 31% of patients with diabetic neuropathic lower extremity ulcers healed within 20 weeks despite standard wound treatment.[5] These physiologic findings of decreased innervation are corroborated by histological documentation of a decreased number of epidermal nerves in diabetic skin.[6–8]

H & E	Hematoxylin & eosin
MNA	4-methoxy-2-naphthylamine
NEP	Neutral endopeptidase
NS	Normal saline
SP	Substance P

Sensory nerves produce tachykinins that signal for vibration, pain, and temperature to the central nervous system, and also induce local proinflammatory effects.[9] Substance P (SP), released by C-fibers in the epidermis and

From the Departments of Surgery[A] and Medicine (Dermatology)[B], University Of Washington, Seattle, Washington; Department of Surgery[C], University of California, San Francisco, California; and Department of Dermatology[D], Northwestern University, Chicago, Illinois.

Reprint requests: Nicole S. Gibran, MD, University of Washington, Department of Surgery, Box 359796, 325 Ninth Avenue, Seattle, WA 98104. Fax: (206)731-3656; Email: nicoleg@u.washington.edu.

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papillary dermis in response to injury, mediates vasodilation[10] and increased vascular permeability,[11] leading to the weal and flare response. SP up-regulates cytokines such as interleukins-1 and -2[12] and adhesion molecules including ICAM-1 and VCAM-1.[13,14] Therefore, decreased nerve numbers in skin from patients with diabetes may result in reduced levels of SP following injury and a diminished inflammatory response. While we have demonstrated improved wound closure kinetics in diabetic mice of wounds treated with topical SP (in press, Journal of Surgical Research), exogenous SP application to diabetic neuropathic skin results in a diminished flare response compared to normal skin.[15] These data suggest that cells in skin or wounds from patients with diabetes may have a diminished response to neuropeptides.

The enzyme neutral endopeptidase (NEP) regulates neuroinflammation through the degradation of proinflammatory mediators such as SP. This membrane-bound metallopeptidase has a high affinity for SP[16] and colocalizes with the SP receptor on endothelial cells, fibroblasts, and keratinocytes.[17,18] NEP competes with the SP receptor to terminate the inflammatory response.[19] NEP enzyme activity in both wounded and unwounded skin samples from human subjects with diabetes mellitus exceeds that found in unwounded skin from normal, age-matched volunteers (in press, Journal of Investigative Dermatology). Therefore, in diabetes, the combined effect of fewer epidermal sensory nerves releasing less SP and increased cutaneous NEP activity may diminish the pro-inflammatory effects of SP that promote healing in the acute wound (Figure 1).

Diabetic mutant (C57BL/6 J-*m*+/+Lepr^{db}; db/db) mice provide a model to study neuroinflammation in wound repair. These animals show characteristics of type 2 diabetes including obesity, hyperglycemia, and insulin resistance.[20] They exhibit impaired wound healing with a delayed and diminished inflammatory response.[21] The db/db mice also exhibit reduced numbers of epidermal nerves,[22] as has been shown in human patients with diabetes.

We hypothesized that increased NEP may retard wound healing and that NEP inhibition would improve closure kinetics in an excisional murine wound model. In this study, we inhibited NEP in a diabetic mouse wound model to determine whether we could improve the response to cutaneous injury as shown by improved time to wound closure.

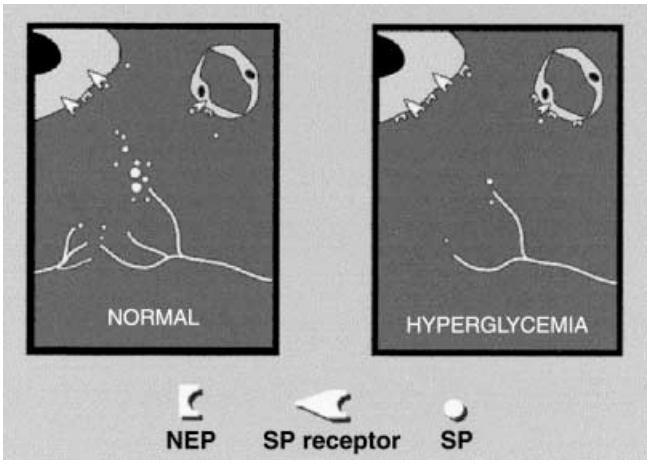

FIGURE 1. Proposed model of neuroinflammation under normal and diabetic conditions.

MATERIALS AND METHODS

Skin samples from male C57BL/KsJ-*m*+/+Lepr^{db} (db/db) and nondiabetic heterozygous (db/–) littermates (Jackson Laboratories, Bar Harbor, ME) ($n = 6$) were weighed and homogenized at −20 °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 μM pepstatin A (Peninsula Laboratories, San Carlos, CA). Protein concentrations were determined using the Pierce Bicinchoninic Acid assay (Pierce, Rockford, IL) to standardize the amount of tissue assayed. NEP enzymatic activity was determined fluorometrically by the degradation of Glutaryl-ala-ala-phe-4-methoxy-2-naphthylamine (Glu-MNA) to 4-methoxy-2-naphthylamine (MNA) as previously described.[23] The NEP activity assay was adapted for evaluation of multiple samples using a 96-well microtiter plate reader. Tissue homogenates (50 μg) were incubated with 80 μM Glu-MNA (Enzymes Systems Products, Livermore, CA) and aminopeptidase M (1 μg/250 μl; Calbiochem, San Diego, CA) with and without thiorphan (25 μM; Sigma Aldrich). The reaction was stopped with 11 mM dithiothreitol (20 μl; Sigma Aldrich). Fluorescent signal was measured in a Cytofluor® Series 4000 fluorometer (340 nm excitation, 420 nm emission). Enzyme activity inhibited by thiorphan (the difference between samples with and without thiorphan) was attributed to NEP and compared to an MNA standard curve. Results are expressed as picomoles of MNA generated per hour per microgram of protein.

Time to wound closure experiment

Both db/db and db/–mice (8–9 weeks old at the initiation of all experiments) were used according to procedures approved by the University of Washington Animal Care Committee. Animals were housed individually and diet consisted of water and standard rodent chow ad libitum. All animals were anesthetized with intraperitoneal ketamine (150 mg/kg) and xylazine (10 mg/kg; Phoenix Pharmaceuticals Inc., St. Joseph, MO). Dorsal hair was shaved

and removed with a depilatory agent (Nair®, Carter Products, NY). The skin was painted with 10% povidone iodine solution and a full-thickness dorsal excisional wound was created with a 6-mm biopsy punch. Wounds were covered with the semiocclusive dressing, Tegaderm® (3M; St. Paul, MN) and 50 μl of treatment solution was infused onto the wound bed. Mice were randomly assigned to receive either normal saline (NS) or the NEP inhibitor thiorphan (10 μM or 25 μM) for 7 days (six mice per treatment group). Digital photographs of the wounds were taken weekly and mice were monitored every 2–3 days until clinical wound closure. Tegaderm® was replaced if the wound was exposed before closure. Wounds were considered closed when they no longer exhibited a moist granulation bed and appeared epithelialized using a hand lens. At wound closure, animals were euthanized with an intraperitoneal injection of pentobarbitol (210 mg/kg; Abbot Laboratories, North Chicago, IL) and the epithelialized wound including a 5-mm margin was excised. Wounds were bisected and fixed in 10% neutral buffered formalin for paraffin embedding or embedded in optimal cutting compound (TissueTek, Torrence, CA) for cryosectioning. Sections were stained with hematoxylin and eosin (H & E) for histological examination.

Time course wound experiment

Wounding was conducted as described above. Forty-two db/db mice were randomized to receive either NS or 25 μM thiorphan topically for 7 days. The unhealed wounds including the underlying dorsal body wall were excised at seven time points following injury: 6, 24, 48, and 72 hours, and 7, 14, and 21 days (three mice per treatment group per time point). After fixation with neutral buffered formalin, the underlying axial tissues were dissected away from the base of the wound and the wound was embedded in paraffin. Sections were cut and stained with H & E.

Wound analysis

Using Adobe Photoshop® 5.0 (Adobe, San Jose, CA), wound edges at day 0 and at the day of closure were delineated on digital photographs by two independent observers. The Image Processing Tool Kit® (Reindeer Games, Asheville, NC) version 2.5 was loaded into the Photoshop® 5.0 plug-ins folder. Scripting to create a set of computerized "actions" was performed according to instructions in the Photoshop® 5.0 manual. Briefly, with an image file open, the "record" button in the Photoshop® action palette can be activated to record command choices that can be saved as a script. The scripts that are recorded and saved can be loaded into the action palette where the functions are performed by pressing the "play" button. Using drawing tools in the program, the boundary between moist granulation tissue and epithelial edge is used to delineate the wound areas; likewise the erythematous perimeter of epidermis is delineated from the uninjured adjacent epidermis. The delineated image is converted to strictly binary data with the inner wound demarcated as black and the outer portion of the image converted to white. Feature measurements (area) were done on binary images in which black pixels were measured and white pixels were not and exported for subsequent analysis in Microsoft® Excel 98. Wound areas were used to calculate degree of wound contraction.

$$\%\ \text{wound contraction} = 100 \times (\text{area}_{\text{Day 0}} - \text{area}_{\text{Day Closed}})/\text{area}_{\text{Day 0}}$$

Histological analysis

Two observers blinded to treatment used a modified histology scale (Table 1)[21] to assign a score to the wound H & E sections. Degree of granulation tissue formation, inflammatory cell infiltration, and fibrosis determined the score.

NEP inhibition in wounds

To determine whether the topical thiorphan inhibited NEP activity in the wounds, we designed experiments to measure NEP enzyme activity in treated and untreated mouse wounds. Because our maximal difference in inflammation occurred on day 3, we chose early time points to assess the effect of thiorphan on wound NEP activity. To have adequate wound tissue to perform enzyme assays, we created 1.5 cm × 1.5 cm full-thickness wounds on four sets of db/db animals (three mice each) using the general procedure described above. Because the wounds were larger than our standard 6 mm punch wounds, we infused 150 μl topical reagent under the

Table 1. Modified histology scale to grade wounds

Score	Criteria
1–3	None to minimal cell accumulation. No granulation tissue formation.
4–6	Immature granulation tissue with few inflammatory cells, fibroblasts, capillaries, and collagen deposition.
7–9	Granulation tissue dominated by inflammatory cells with few fibroblasts or capillaries.
10–12	Vascular granulation tissue dominated by fibroblasts, capillaries, and extensive collagen deposition.
13–15	Immature scar dominated by parallel fibroblasts.

semipermeable dressing. Group 1 was treated with NS on day 0 and euthanized 1 hour after treatment for wound harvesting; Group 2 was treated with thiorphan (25 μM) on day 0 and euthanized 1 hour after treatment for wound harvesting; Group 3 was treated with NS on days 0 and 1 and euthanized 1 hour after the second treatment for wound harvesting; Group 4 was treated with thiorphan (25 μM) on days 0 and 1 and euthanized 1 hour after the second treatment for wound harvesting. The wounds were processed for NEP enzyme assay as described above.

Statistical analysis

Data are expressed as means ± standard deviation. NEP activity and wound closure data were analyzed by ANOVA. Probability values less than 0.05 were accepted as statistically significant.

RESULTS

NEP enzyme activity in murine skin is summarized in Figure 2. NEP activity from unwounded db/db murine skin (20.6 ± 4.5 pmol MNA/hour/μg protein) significantly exceeds db/–skin (7.9 ± 2.7 pmol MNA/hour/μg protein; $p = 0.02$). These results corroborate our observations of elevated NEP enzyme activity in skin samples from human subjects with diabetes mellitus. Treatment of the wounds with thiorphan decreased NEP activity in the wound on days 0 and 1 compared to NS-treated wounds. Because there was no difference in NEP activity in the NS-treated wounds on day 0 (1.02 pmols MNA/hour/μg) and day 1 (1.44 pmols MNA/hour/μg) nor in the thiorphan-treated wounds on day 0 (0.74 pmols MNA/hr/μg) and day 1 (0.45 pmols MNA/hour/μg), the six wounds (three for day 0 and three for day 1) in the treated and control groups were pooled for statistical evaluation. Thiorphan-treated wounds (0.6 pmols MNA/hour/μg) had significantly lower NEP activity than NS-treated wounds (1.23 pmols MNA/hour/μg; $p = 0.02$).

Wound closure studies

Mutant db/db mice weighed between 30 and 40 g and db/– mice weighed between 20 and 30 g. All mice that survived anesthesia tolerated the wounding procedure and treatment course with no change in food consumption or weight.

The average original wound area for db/–mice was 32.5 ± 7.4 mm^2 and for db/db mice was 39.4 ± 8.3 mm^2 ($p = 0.01$). The mice in the db/db treatment groups had no significant difference in original wound areas ($p = 0.27$); the area of wounds for db/db mice treated with NS was 43.5 ± 8.2 mm^2, with 10 μM thiorphan was 38.4 ± 9.1 mm^2, and with 25 μM thiorphan was 35.2 ± 7.0 mm^2.

The db/db murine wounds treated with 25 μM thiorphan (18.0 ± 0 days; $p = 0.03$) closed significantly faster (Figure 3) than wounds treated with NS (23.5 ± 3.7 days) or 10 μM thiorphan (26.8 ± 7.2 days). NEP inhibition did not alter wound closure kinetics in the db/–mice. NS-treated db/–wounds closed in 13.6 ± 1.1 days, 10 μM thiorphan in 13.6 ± 1.1 days, and 25 μM thiorphan in 14.0 ± 0 days ($p = 0.74$).

Contraction in healed wounds (Figure 4) in the NS-treated db/–mice (83% ± 4) exceeded the db/db mice (73% ± 4; $p = 0.02$). These data confirm previous reports that contraction in the db/–mice contributes more to wound closure than in the db/db mice. No difference in wound size could be shown between the three db/db treatment groups at closure, suggesting that enhanced epithelialization contributed to the healing process in the treated wounds that healed faster (Figure 5). The degree of

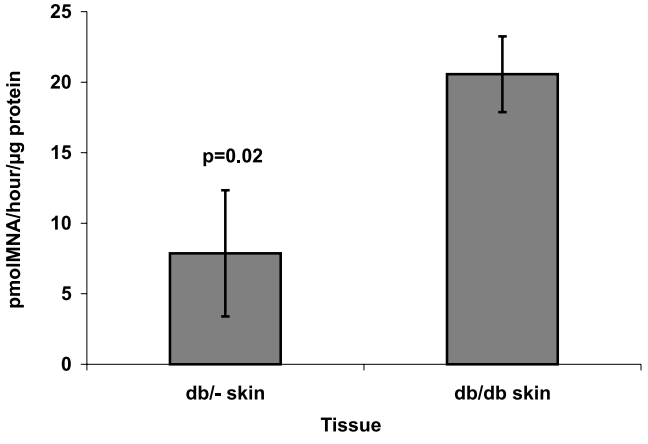

FIGURE 2. Average NEP activity in unwounded db/–skin is significantly less than in db/db murine skin ($p = 0.02$). Standard error bars represent one standard deviation.

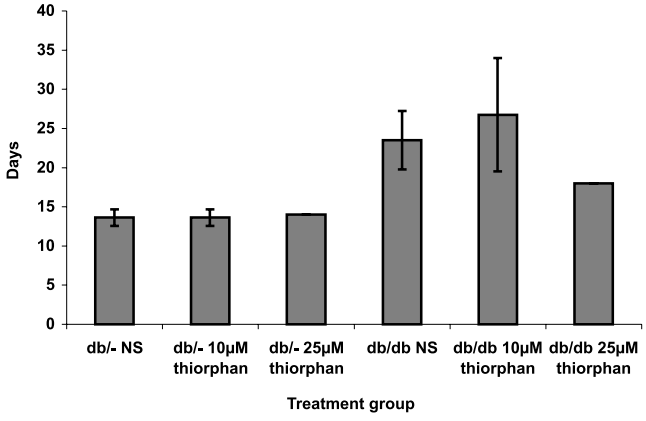

FIGURE 3. Average time to wound closure for db/–mice is not changed with thiorphan treatment. However, 25 μM thiorphan significantly ($p = 0.03$) shortens the time to closure in db/db mice compared with those treated with NS or with 10 μM thiorphan. Standard error bars represent one standard deviation.

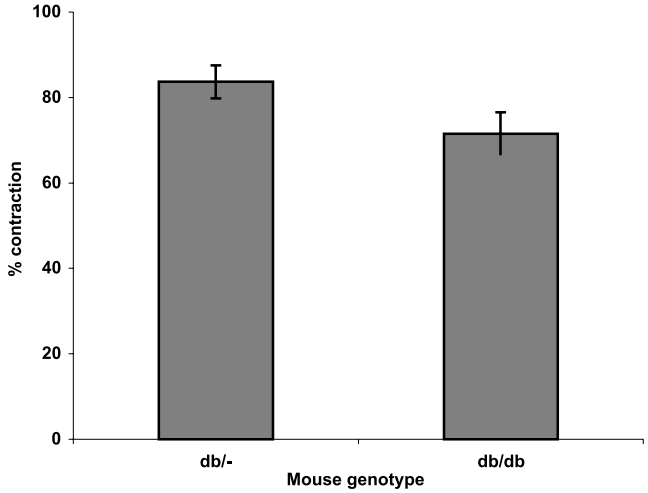

FIGURE 4. Average percentage wound contraction at time of closure is significantly greater in the db/–mice than in the db/db mice ($p = 0.02$). Standard error bars represent one standard deviation.

contraction of wounds for db/db mice treated with NS was 73% ± 9, 10 μM thiorphan was 67% ± 15, and 25 μM thiorphan was 65% ± 23 ($p = 0.73$). The degree of contraction in wounds for db/–mice treated with NS was 83% ± 4, 10 μM thiorphan was 81% ± 15, and 25 μM thiorphan was 88% ± 3 ($p = 0.48$).

Histological evaluation of healed wounds showed no significant difference in inflammation or granulation tissue between db/db treatment groups using a semiquantitative scoring system with two observers blinded to treatment. While inflammation in the thiorphan-treated wounds appeared greater than the NS-treated wounds at 48 hours (Figure 6) and the mean histological score (Table 2) for thiorphan-treated early wounds (48 hours) was greater than for the wounds treated with NS, these evaluations were not significantly different.

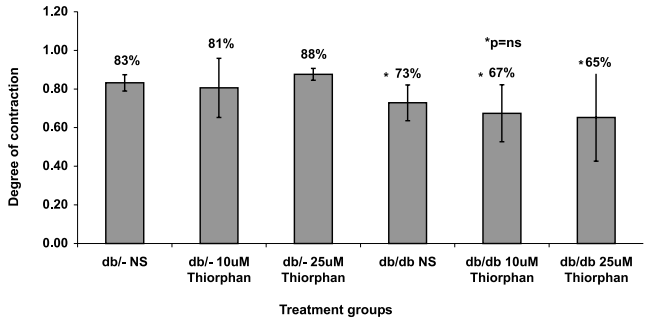

FIGURE 5. Average percentage wound contraction values show that thiorphan did not change the rate of wound contraction in either the db/–or db/db mice. Standard error bars represent one standard deviation.

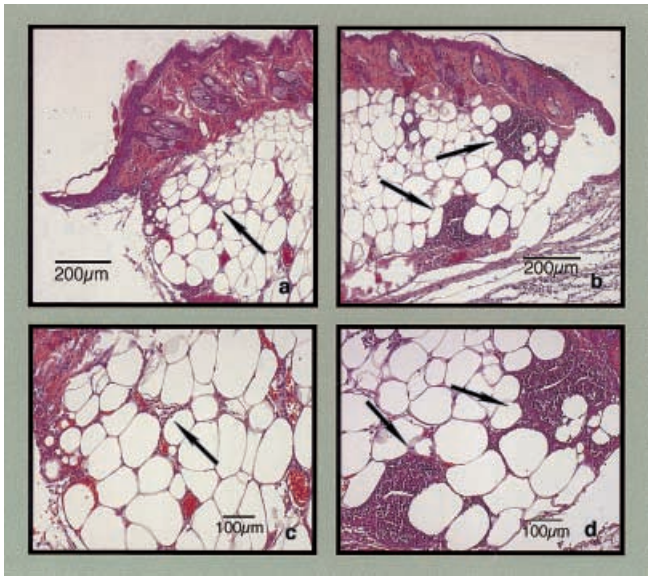

FIGURE 6. H & E stained images of db/db mouse wounds collected at 48 hours postinjury. (a and c) Wound treated with NS and (b and d) wound treated with 25 μM thiorphan. Arrows indicate that inflammation is greater in the thiorphan-treated wounds.

DISCUSSION

Our observation of elevated NEP activity in unwounded db/db mouse skin correlate with our previous studies showing increased NEP activity in unwounded skin from patients with diabetes mellitus compared with normal volunteers.[24] These data suggest that in patients with diabetes mellitus, cutaneous SP availability may be decreased constitutively through enhanced enzymatic degradation. Our finding of increased NEP enzyme activity in diabetic mutant mice supports the use of this mouse model for studying aberrant neuroinflammation in impaired wound healing.

Increased NEP in skin from human subjects with diabetes and db/db mice may result from one or more of several abnormal metabolic conditions associated with diabetes mellitus. Because NEP contains glycosylation sites that determine transport of the enzyme to the cell surface and enzyme activity, hyperglycemia and protein glycation may impact NEP activity levels.[25] Alternatively, glucocorticoids increase NEP mRNA expression and enzyme activity in transformed human tracheal epithelial cells[26] and may be increased in mutant diabetic mice.[27]

By showing improvement in wound closure kinetics through NEP inhibition, we corroborate previous studies in our lab showing improved wound closure kinetics in db/db wounds treated with topical SP application (submitted for publication). Together these data support our hypothesis that SP has a role in the response to injury. The observation that 10μM thiorphan increased time to wound closure (p = ns) compared to NS treatment may reflect the

Table 2. Mean inflammatory scores for wounds in db/db mice treated with thiorphan

Time point (hours)	Treatment	Inflammatory score
6	Normal saline	2.5 ± 0.9*
	25 μM thiorphan	3.5 ± 1.1
24	Normal saline	5.8 ± 1.2
	25 μM thiorphan	6.0 ± 0.9
48	Normal saline	7.2 ± 1.0
	25 μM thiorphan	7.4 ± 0.5
72	Normal saline	6.5 ± 1.1
	25 μM thiorphan	7.3 ± 0.3

* Value indicates mean ± SD.

fact that this dose had no effect on cellular response to injury.

Whether NEP-induced improvement in wound closure was due to wound contraction or epithelialization was determined by the measurement of the sizes of the healed wounds. Sizes of the healed db/db murine wounds in all treatment groups were comparable and there was no difference in degree of contraction, suggesting that the beneficial effect of NEP on wound closure kinetics resulted from augmented epithelialization rather than from wound contraction. Because SP induces keratinocyte proliferation and migration,[28] increased SP availability may directly affect epithelialization in this model of delayed healing.

Cell signaling pathways in normal wound healing begin with a robust inflammatory response. Previous studies confirm that cellular immunity is diminished in db/db mice[29] and inflammation is delayed in cutaneous response to injury.[21] In our study, histological scores for thiorphan-treated wounds collected during the first 72 hours post-wounding exceeded the scores for NS treatment, suggesting that NEP inhibition augments the inflammatory response. However, these results did not reach statistical significance, possibly due to the small number of animals in each treatment group ($n = 3$) resulting in a Type 2 error. An enhanced inflammatory response through NEP inhibition may provide a mechanism for therapeutically improving response to injury in the setting of diabetes.

We have presumed in these studies that NEP inhibition implicates a role for SP in wound repair. However, other possible substrates include bradykinin, calcitonin gene related peptide, atrial natiuretic factor, enkephalins, and endothelins;[16] these cytokines can also have proinflammatory effects on target cells. NEP has been reported to reduce the effects of platelet-derived growth factor-AB and transforming growth factor-β1 on immunocyte cell shape.[30] While the authors showed that NEP does not directly cleave either growth factor, they did not define a definitive mechanism for the NEP effect. Therefore, NEP may affect cellular responses that do not involve SP-induced inflammation during wound repair.

Alternative explanations for the thiorphan-induced improvement in wound healing must also be considered. Thiorphan is a specific NEP inhibitor, unlike phosphoramidon, which also inhibits endothelin converting enzyme.[31] We demonstrated reduced NEP enzyme activity in wounds treated with thiorphan. However, thiorphan has recently been shown to bind other metalloproteases including matrix metalloproteinase 7 (matrilysin).[32] Matrilysin expression is up-regulated in corneal basal epithelial cells after wounding and has been speculated to be involved in epithelial migration and proliferation.[33] Therefore, an interaction between matrilysin and thiorphan may have contributed to our observed improvement in wound closure kinetics.

Future studies must elucidate the mechanisms by which NEP activity is increased in diabetic skin and verify the means by which NEP inhibition might improve cutaneous response to injury.

In summary, NEP enzyme activity in mutant diabetic murine skin significantly exceeds that found in nondiabetic murine skin. These results correlate with previous studies characterizing elevated NEP activity in human diabetic skin compared with skin from normal age-matched volunteers. The NEP inhibitor thiorphan improved wound closure kinetics in diabetic mice and appeared to enhance the inflammatory response in diabetic mouse wounds.

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