Topical Naltrexone as Treatment for Type 2 Diabetic Cutaneous Wounds

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Objective: Type 2 diabetes (T2D) is associated with impaired cutaneous wound healing and can result in ulceration, infection, and/or amputation. More than 25 million people in the United States have T2D and are vulnerable to epithelial-related complications. Current therapies are limited in their efficacy. New treatments for full-thickness cutaneous wounds that focus on underlying diabetic pathways are needed.

Approach: Topical application of the opioid receptor antagonist naltrexone (NTX) dissolved in cream reverses delayed wound closure in type 1 diabetic rat by the acceleration of reepithelialization and enhancement of angiogenesis and remodeling. NTX blocks the opioid growth factor (OGF)–OGF receptor (OGFr) axis and upregulates DNA synthesis and cell proliferation. To investigate whether NTX is an effective therapy for T2D wound closure, genetically obese mice (*db/db*) and normal C57Bl/6J mice received full-thickness cutaneous wounds. Wounds (5 mm in diameter) were treated topically three times daily with 10^{-5} M NTX or sterile saline dissolved in cream and photographed every 2 days.

Results: Wounds in *db/db* mice treated with saline were 11–92% larger than those in normal mice throughout the 2-week observation. Topical NTX therapy in T2D mice reduced the residual wound size by 13–30% between days 8 and 14 relative to diabetic mice receiving saline. Reepithelialization and DNA synthesis, as analyzed by epithelial thickness and BrdU labeling indexes, respectively, were accelerated in NTX-treated wounds.

Innovation and Conclusion: These data suggest that the OGF-OGFr axis plays a role in epithelial-related complications of T2D and that blockade of this pathway by NTX may be an effective treatment for wound repair.

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INTRODUCTION

TYPE 2 DIABETES (T2D) accounts for 90–95% of the 25 million individuals in the United States with diabetes.¹ T2D is associated with defects in insulin secretion and insulin resistance.^{1–3} The annual cost of treatment and maintenance of persons with diabetes approaches \$245 billion in the United States and is substantially more if the treatment of prediabetic conditions is included.⁴ In addition to the treatment of the disease directly, a number of com-

plications associated with delayed epithelialization are prevalent in diabetes; treatment of the complications accounts for 18% of the total expenditure,^{3,4} with impaired wound healing being a major drawback. Impairments in cutaneous wound healing can result in chronic lesions, ulcers, epithelial erosion, and amputation of the extremities.^{1–4} It is estimated that nearly 50% of the 170 million people worldwide with diabetes will experience delayed cutaneous wound healing.^{1–5}

Wound healing is a dynamic process that culminates in the restoration of the anatomical function and continuity in tissue. The process of wound closure is a continuum of overlapping phases, including homeostasis, inflammation, proliferation, and formation of granulation tissue during remodeling.^{6–8} Each phase is initiated by cytokines and growth factors and is compromised in diabetes, with delays in cell replication being involved in each step.^{6–10}

The opioid antagonist naltrexone (NTX) topically applied to full-thickness wounds in type 1 diabetic animals accelerates wound repair.^{11–13} NTX lacks intrinsic biological activity but blocks interactions between opioid peptides and their receptors.^{14,15} The endogenous opioid peptide [Met⁵ enkephalin], also termed opioid growth factor (OGF), regulates cell growth. OGF inhibits cell proliferation through interactions with the OGF receptor (OGFr), and this regulatory pathway is a tonically active growth determinant in wound healing.^{16–18} NTX is known to block OGF-OGFr interaction and to increase cell proliferation if the duration of receptor blockade is complete.^{18–24} Furthermore, neutralization of OGF by antibodies to peptide or a decrease in OGFr by antisense cDNA or siRNA¹⁸ increases DNA synthesis and stimulates cell proliferation. NTX administration at high doses produces a continuous blockade of opioid receptors that stimulates the secretion of peptide and receptor, but because OGFr is blocked by NTX and unable to interact with OGF, there is an increase in DNA synthesis, cell proliferation, and full-thickness wound healing.^{11,12,23}

In this study, the effects of topical application of NTX to full-thickness cutaneous wounds in a genetic mouse model of T2D were studied.

CLINICAL PROBLEM ADDRESSED

A major complication of diabetes is delayed closure of full-thickness cutaneous wounds. The delayed closure results in increased risk of infection, ulceration, and possible amputation of an extremity. NTX is a safe and easily administered treatment that enhances wound closure by targeting the underlying disease-modifying pathways in T2D.

MATERIALS AND METHODS

Animals

The animals used in these experiments were *db/db* mice (strain B6.BKS(D)-Lepr^{db}/J) and normal C57BL/6J mice from Jackson Laboratories. The *db/db* strain of mice is homozygous for the spontaneous diabetes mutation and the mice become

obese in 3–4 weeks, thereby providing a useful model of T2D. These animals display elevated blood glucose levels in 4–8 weeks and are polyphagic, polydipsic, and polyuric. The *db/db* mouse can manifest myocardial disease, peripheral neuropathy, and delays in wound healing. Animals used in this study were between 8 and 12 weeks of age and were individually housed in an environmentally controlled room (temperature 21°C ± 0.5°C, humidity 50% ± 10%, 10–15 air changes per hour, 12 h light/dark schedule with no twilight). Mice were provided water and food (2018 Global Rodent Diet; Teklad) *ad libitum*.

Body weight and blood glucose measurements

Glucose measurements and body weights were obtained at the time of surgical wounding. Blood glucose levels > 340 mg/dL indicated a hyperglycemic state, and animals were randomized to the T2D group. *Db/db* mice that had blood glucose levels > 600 mg/dL and were not feeding, drinking, or appeared lethargic were excluded from the study.

Wound surgery and splinting

Before the surgery, the dorsum (2 × 5 cm area) of the mouse was shaved using an electric shaver followed by the application of Nair[®] to remove all hair. Animals were anesthetized by intraperitoneal injections (0.1 mL solution) of ketamine (60 mg/kg) (Ketaject[®]; Phoenix Pharmaceutical, Inc.), xylazine (10 mg/kg) (TranquiVed[®] Injections; Vedco, Inc.), and acepromazine (1 mg/kg) (Acepromazine Maleate Injection; Vedco, Inc.). Two 5-mm full-thickness excisional wounds were created on the dorsum of each animal; one was positioned on the midline just cranial to the thoracic kyphosis and the second was positioned just caudal to the thoracic kyphosis. Wounds were scored using a biopsy punch (Acuderm, Inc.) and dissected with small surgical scissors to ensure standardized wounds. After the surgery, wounds were treated with an antiseptic. Surgeries were performed between 08:00 and 11:00 h to reduce the effects of diurnal rhythm on healing. Strapping tape splints were applied around each individual wound. A hole in the middle of the splint was created by scoring the strapping tape with a 6-mm-diameter Acupunch; the splints were reinforced with Krazy Glue[®] at each corner.²⁵ If the splint had to be replaced more than three times during the 20-day study, the animal was removed from data analysis.

NTX administration

Approximately 0.1 mL of NTX or control therapy was administered at each treatment time. NTX, dissolved in Neutrogena moisturizing cream (final

concentration of 10^{-5} M NTX cream) or saline (0.5 mL saline dissolved in moisturizing cream, vehicle), was topically applied immediately after the surgery and at 09:00, 13:00, and 18:00 h daily for 14 days. Each animal had one NTX-treated wound and one control-treated wound; the treatment was randomized for cranial or caudal wounds.

Photography and areal analyses

Residual wound size was monitored with a digital camera mounted on a tripod located ~ 15 cm above the animal. To calibrate measurements, a ruler was photographed adjacent to the wounds. For photography, the animals were sedated in a regulated vaporizer with a 3% isoflurane–oxygen mixture. Photographs were taken immediately after the surgery (day 0) and every other day for 2 weeks. Areal analysis of each wound was performed using the Image ProPlus 6.2 (Media Cybernetics, Inc.) software, and the percent area of residual defect was calculated.

Histopathology and DNA synthesis

The animals were euthanized by intraperitoneal injection of 0.2 mL Euthasol® (Virbac AH, Inc.). Following euthanasia, a 2 cm² region of skin surrounding each wound was dissected and removed; further trimming was required to ensure that granulation tissue was sectioned. Specimens were fixed, processed in paraffin, and sectioned (10 μ m). Epithelial thickness was assessed in hematoxylin–eosin-stained sections of newly deposited granulation tissue collected 20 days after the surgery from mice in each group. For comparison, three unwounded normal and three unwounded *db/db* mice were euthanized, and tissue was collected from the dorsal midline to establish baseline levels of epithelial thickness. Tissue specimens were visualized with 20 $\times$ magnification on an Olympus BH2-RFCA microscope and photographed using the Spot Advanced software. Epithelium was measured over the granulation tissue using the Image Pro Plus 6.2 (Media Cybernetics, Inc.) software.

To assess cell replication in the wounded region, mice were injected with bromodeoxyuridine (BrdU) and proliferation was examined in the basal epithelium located superior to the newly deposited granulation tissue. Six days after wounding, *db/db* ($n=9$) and C57BL/6J ($n=8$) mice were injected twice with 0.2 mL of BrdU at 6 and 3 h before euthanasia. Mice were euthanized and the tissue was harvested and sectioned (10 μ m). Immunohistochemistry was completed by staining with the anti-BrdU antibody (Invitrogen; 1:200) and mouse monoclonal secondary antibody (Invitrogen; 1:1,000). BrdU labeling indexes were determined

as the percentage of positive BrdU-labeled basal epithelial cells per total basal epithelial cells superficial to the wound site.

Statistical analysis

Body weights and glucose measurements, as well as epithelial thicknesses in unwounded animals, were evaluated using the Student's two-tailed *t*-test. Residual wound areas, epithelial thicknesses, and BrdU labeling indexes were analyzed using analysis of variance with subsequent planned comparisons made using Newman–Keuls tests.

RESULTS

Body weight and blood glucose measurements

Normal C57BL/6J mice weighed 26.1 ± 0.6 g and the *db/db* mice weighed 47.8 ± 0.4 g, approximately a 1.8-fold difference in body weight (Fig. 1A). Diabetic mice had blood glucose levels > 435 mg/dL at the time of surgery, whereas control mice had a mean of 245 ± 16 mg/dL (Fig. 1B).

Full-thickness wound closure

In the first set of experiments, normal and *db/db* animals were wounded, splinted, and their residual wound areas photographed over a 14-day period (Figs. 2 and 3). Normal mice receiving either saline (normal + vehicle) or NTX (normal + NTX)

Figure 1. (A) Body weights (g) and (B) glucose levels (mg/dL) of diabetic (*db/db*) and normal adult male mice at the time of surgery. Values represent means $\pm$ SEM for 10–12 mice per group. Significantly different from normal values at *** $p < 0.001$.

Figure 2. Photographs of full-thickness wounds following the application of topical NTX to normal and type 2 diabetic (*db/db*) mice. Wounds (5 mm in diameter) were created on the dorsum and treated three times daily with either 10^{-5} M NTX (NTX) or saline (vehicle) dissolved in Neutrogena moisturizing cream. Photographs were taken immediately after the surgery (day 0) and on days 4, 8, and 12. Bar = 5 mm. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

dissolved in moisturizing cream had complete closure within the 2-week observation period. Residual wound defects were comparable at each time point for both the groups (data not shown). In contrast, T2D mice with wounds treated with

moisturizing cream alone had wounds that were only 50% closed by day 14. Diabetic mice treated topically with NTX had significantly smaller residual wound areas relative to the *db/db* + vehicle animals at all time points (Fig. 3). Although the residual surgical wound was larger in the *db/db* mice relative to normal mice, NTX treatment of wounds on *db/db* mice reduced the residual wound size to 35% of baseline by day 14 in comparison to the 50% residual wound measured in vehicle-treated T2D mice at this time point (Fig. 3). The *db/db* + NTX mice had significantly smaller wounds compared with *db/db* + vehicle mice on days 6, 8, 10, 12, and 14 (Figs. 2 and 3), with residual wounds that were 11–92% larger than those in normal mice. NTX therapy of T2D mice reduced the residual wound size by 13–30% between days 8 and 14 compared with *db/db* + vehicle mice (Fig. 3).

The rate of wound closure was evaluated by calculating the mean area of closure per day. Between days 2 and 4, NTX-treated *db/db* mice and normal mice had closure rates in excess of 4 mm^2 per day in comparison to normal control mice with 3.25 mm^2 and diabetic mice receiving saline showing only 1.75 mm^2 per day.

Morphological evaluation of reepithelialization

Tissue sections were stained with H&E to evaluate the morphology of wound closure (Fig. 4). The thickness of the epithelium was measured in unwounded skin, as well as in regions superficial to the granulation tissue collected from mice euthanized 20 days after the surgery. The thickness of epithelium in unwounded skin differed significantly between

Figure 3. Full-thickness wound closure following the topical application of NTX to normal and *db/db* mice. Residual wound areas (%) following surgical wounding on the dorsum of normal mice receiving saline dissolved in Neutrogena moisturizing cream (normal + vehicle) or T2D mice (*db/db*) receiving either saline dissolved in Neutrogena moisturizing cream (*db/db* + vehicle) or 10^{-5} M NTX dissolved in Neutrogena moisturizing cream (*db/db* + NTX). Wounds were treated three times daily for 14 days. Significantly different between *db/db* + vehicle and normal + vehicle measurements at *** $p < 0.001$. Significantly different between *db/db* + NTX and *db/db* + vehicle at + $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Figure 4. Epithelialization of cutaneous wounds in normal and diabetic (*db/db*) mice after 14 days of treatment with topical application three times daily of NTX or saline dissolved in Neutrogena moisturizing cream. **(A)** Histogram of epithelial thickness (μm) in normal and *db/db* unwounded skin. Values represent means $\pm$ SEM. Significantly different from normal values at *** $p < 0.001$. **(B)** Histograms of epithelial thickness (μm) over the granulation tissue in the wound bed in normal and *db/db* wounded skin treated with NTX or saline. Values represent means $\pm$ SEM. Data from NTX-treated wounds were compared with vehicle-treated wounds for normal or *db/db* mice. Significantly different from vehicle-treated specimens at *** $p < 0.001$. **(C)** Photomicrographs of epithelium stained with hematoxylin and eosin; bar = 25 μm . Tissues from normal and *db/db* mice were treated with NTX or vehicle for 14 days. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

normal and *db/db* mice. Normal mice had epithelium that was $\sim 17 \mu\text{m}$ thick, whereas *db/db* mice had epithelium that was $\sim 43 \mu\text{m}$ thick (Fig. 4A).

The thickness of epithelium in sections of wounded skin collected 20 days after the surgery from normal or *db/db* mice, treated with either NTX or vehicle (Fig. 4B, C), revealed that wounds

treated with NTX in normal mice had 1.7-fold thicker epithelium in comparison to those from normal mice receiving control cream. The epithelium of wounds treated with NTX in *db/db* mice was 1.5-fold thicker in comparison to the measurements of epithelium of control-treated wounds in diabetic mice.

Figure 5. Proliferation of cells in the basal epithelial layer in renewed skin over the granulation tissue of closed wounds; normal and *db/db* mice were treated with topical applications (three times daily) of NTX or saline dissolved in Neutrogena moisturizing cream for 14 days. **(A)** DNA labeling indexes calculated from the number of BrdU-positive basal cells relative to the total number of basal cells in **(B)** sections of skin treated topically with either NTX or saline dissolved in cream. Values represent mean $\pm$ SEM. Significantly different from normal + vehicle group at *** $p < 0.001$. Labeling indexes in NTX-treated diabetic (*db/db* + NTX) skin were significantly different from measurements in saline-treated tissue from T2D mice (*db/db* + vehicle) at *** $p < 0.001$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

The epithelium surrounding the wound area after 6 days of surgery was also evaluated. No measurements could be reliably taken in *db/db* + vehicle mice; epithelial thickness was less than two layers thick in normal mice as well.

The evaluation of BrdU labeling indexes for normal animals revealed that control-treated and NTX-treated wounds had comparable proliferation rates of ~43% in the basal epithelium. Wounds from diabetic mice treated with NTX had DNA synthesis rates of 52% in comparison to BrdU incorporation in wounds treated with control cream.

Db/db + vehicle animals had a proliferative index of ~19% (Fig. 5), a 64% reduction from mice treated with NTX, and 44% of levels recorded in normal animals receiving control cream.

DISCUSSION

A major complication of T2D, as well as T1D, is a delay in reepithelialization of skin that has been abraded or wounded. Diabetic ulcers are common, affecting more than 50% of persons with diabetes at one time or another. In addition to the pain and

disability afforded to the individual, delayed wound closure renders diabetic patients vulnerable to more serious complications, such as infection and amputation.¹⁻⁴ In this study, a genetic mouse model of T2D was utilized to determine if NTX, an opioid receptor antagonist, applied topically, could accelerate the closure of full-thickness cutaneous wounds. Previous studies have reported that topical NTX accelerated epithelialization in corneal surface wounds of streptozotocin-induced type 1 diabetic rats.²⁶ Ocular surface defects are an epithelial-related complication of diabetes that can lead to recurrent erosion and blindness. Topical application of NTX restored the corneal surface in less time, with 20–42% faster healing rates than reported in diabetic rats receiving only vehicle.²⁶ Moreover, NTX applied in moisturizing cream reduced the residual wound size in type 1 diabetic rats,¹¹ increased angiogenesis,¹² and accelerated the processes associated with wound remodeling.¹³

The present investigation utilized a mouse model of T2D and revealed that topical NTX accelerated wound closure. These data suggest that NTX is a novel therapy for nonhealing ulcers in individuals with T2D, a population approaching nearly 23 million in the United States.¹ The present study extends and confirms our previous work in several ways, thereby confirming the effects of NTX to accelerate wound closure, an epithelial-related complication associated with both T1D and T2D. The *db/db* mouse represents a genetic animal with deletions in the *Lepr* gene. The present data provide comparisons between models of diabetes in mice and rats, between genetically driven and chemically induced hyperglycemic animal models, as well as complications from two different metabolically driven disorders. Mice also have different properties associated with wound closure and heal with a combination of epithelialization and contraction. To offset confounding effects of contraction, the excisional wounds in the present study were splinted to prevent skin contraction and to allow healing through granulation tissue deposition and reepithelialization.²⁷⁻²⁹ This model resulted in uniform closure of cutaneous wounds by decreasing the amount of variation due to contraction and wound dressings. Splinting minimized contraction but did not prevent it completely. A thick layer of subcutaneous fat is present on the dorsum of *db/db* mice and serves to maintain taut skin. C57Bl/6J mice lack this layer of fat, allowing contraction to more likely contribute to wound closure than in obese *db/db* mice.

The present study revealed that *db/db* mice had delayed rates of closure of full-thickness circular

wounds in comparison to normal mice. Topical applications of NTX accelerated wound closure in *db/db* mice compared with healing rates for *db/db* mice treated with control moisturizing cream. NTX application to *db/db* mice did not, however, increase wound healing rates to those of normal mice, and by day 14 after the surgery, residual wounds were still apparent in both the groups of *db/db* mice, whereas wounds in normal mice were closed.

Investigation into the optimal treatment regimen was initiated by applying NTX either once or thrice daily (data not shown). One application of NTX resulted in wounds that closed at a similar rate to, or even more slowly, than untreated diabetic wounds. Whether a single application of NTX is invoking a short-term receptor blockade, which results in upregulation of the OGF-OGFr axis and subsequent inhibition of cellular proliferation, is unknown. Additional studies are required to determine whether the topical administration of NTX confers a short-term opioid receptor blockade that is capable of modulating the OGF-OGFr axis.

The mechanism of action for topical NTX application to cutaneous wounds in T2D involved reepithelialization and stimulated DNA synthesis. BrdU labeling of wounded regions in *db/db* mice treated with NTX had elevated labeling indexes, whereas DNA labeling was not elevated in normal mice treated with NTX, suggesting that the timetable for cell proliferation occurs early in the healing process. DNA synthesis may commence 24 h postinjury (38), and thus, the effects of NTX on normal healing were not evident in tissue collected at 1 week postsurgery.

Epithelialization was altered by NTX treatment in both normal and diabetic wounds at day 20. The thickness of the epithelium of the restructured wound was increased in animals treated with NTX relative to that measured in mice receiving topical saline.

Collectively, these data support and extend observations that in a mouse model of T2D, topical NTX application blocks the OGF-OGFr axis and accelerates delayed wound closure. Thus, topical NTX advances wound care and serves as an effective treatment of epithelial-related complications associated with diabetes.

INNOVATION

Topical application of an opioid antagonist for treatment of cutaneous wounds advances wound care in diabetes.

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Jessica Immonen, MS, PhD, completed this work as a portion of her doctoral thesis in anatomy at the Penn State University College of Medicine. Dr. Immonen is currently an Assistant Professor of Anatomy, Rocky Mountain University of Health Professions.

KEY FINDINGS

- Full-thickness cutaneous wounds in T2D mice heal more slowly than normal mice.
- Topical application of NTX accelerates the closure of full-thickness cutaneous wounds in T2D mice relative to saline treatment.
- Topical NTX increases DNA synthesis in basal epithelial cells of the skin.
- Blockade of the OGF-OGFr axis with NTX targets an underlying pathway in diabetes.
- Topical NTX is a novel therapy for complications in diabetes related to reepithelialization.

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Abbreviations and Acronyms

NTX = naltrexone
OGF = opioid growth factor
OGFr = opioid growth factor receptor
T2D = type 2 diabetes