Gene Therapy of Endothelial Nitric Oxide Synthase and Manganese Superoxide Dismutase Restores Delayed Wound Healing in Type 1 Diabetic Mice

Jian-Dong Luo, MD, PhD; Ying-Ying Wang, MD; Wei-Ling Fu, MD, PhD; Jun Wu, MD, PhD; Alex F. Chen, MD, PhD

Background—Nitric oxide (NO) deficiency contributes to diabetic wound healing impairment. The present study tested the hypothesis that increased cutaneous superoxide (O$_2^-$) levels in type 1 diabetic mice cause NO deficiency and delayed wound healing.

Methods and Results—Wound healing was markedly delayed in streptozotocin-induced type 1 diabetic mice compared with the normal controls. There were significantly reduced levels of endothelial NO synthase (eNOS) protein and constitutive NOS activity in diabetic wounds, whereas O$_2^-$ levels were markedly increased. A single regimen of cutaneous gene therapy of eNOS or manganese superoxide dismutase (MnSOD) restored such healing delay, with a concomitant suppression of wound O$_2^-$ levels and augmentation of both eNOS protein and constitutive NOS activity. Gene therapy of MnSOD also increased cutaneous MnSOD activity. Cutaneous O$_2^-$ levels were also increased in Ins2Akita diabetic mice. In vitro glucose treatment of cutaneous tissues from normal mice for 24 hours increased O$_2^-$ levels in a concentration-dependent manner. The enhanced cutaneous O$_2^-$ levels induced by high glucose in both normal and diabetic mice were abolished by the NADPH oxidase inhibitor apocynin and the protein kinase C inhibitor chelerythrine. Furthermore, ex vivo gene transfer of dominant-negative HA-tagged N17Rac1, which inhibits NADPH oxidase subunit Rac1, significantly inhibited cutaneous O$_2^-$ formation induced by high glucose in both normal and Ins2Akita diabetic mice.

Conclusions—These results indicate that hyperglycemia augments cutaneous O$_2^-$ levels, at least in part, via NADPH oxidase and protein kinase C pathways, resulting in impaired wound healing in type 1 diabetic mice. Gene therapy strategies aimed at restoring cutaneous NO bioavailability may provide an effective means to ameliorate delayed diabetic wound healing. (Circulation. 2004;110:2484-2493.)

Key Words: wound healing n nitric oxide n superoxides n diabetes mellitus n gene therapy

Wound healing impairment in diabetic patients represents a particularly challenging clinical problem for which there is currently no efficacious treatment regimen.¹ The etiology of delayed diabetic wound healing is multifactorial, and vasculopathy and neuropathy are major contributors.¹ Diabetes is characterized by chronic hyperglycemia associated with significant vasculopathy.² Sustained hyperglycemia is known to increase vascular superoxide (O$_2^-$) production, which inactivates nitric oxide (NO) and causes vascular dysfunction in diabetes.³⁻⁵

Compelling evidence indicates that NO plays an integral role in normal wound repairs.⁶⁻⁷ NO promotes processes central to wound healing, including angiogenesis⁸⁻¹⁰ and migration and proliferation of fibroblasts,¹¹ epithelial cells,¹² endothelial cells,⁸⁻⁹ and keratinocytes.¹⁰ Consequently, a NO deficiency contributes to wound healing impairment. The NO synthase (NOS) inhibitors, when applied to the wound surface¹³ or given systemically,¹⁴ retard wound healing. In contrast, a systemic administration of the NO precursor L-arginine improves wound repair in normal individuals¹⁵ and in aged individuals with impaired healing.¹⁶ In agreement with these findings, recent studies with targeted disruption of NOS genes have revealed that the excisional wound closure is delayed by 30% in both endothelial NOS (eNOS) and inducible NOS (iNOS)–knockout mice compared with the wild-type littermates.¹⁷,¹⁸ Conversely, adenoviral vector–mediated gene transfer of iNOS to the wound site of iNOS-knockout mice completely reversed the delayed healing.¹⁸

In streptozotocin-induced mice (a model of type 1 diabetes), impaired wound healing is paralleled by both decreased
wound NOS expression and NO levels,19 and l-arginine improves such healing delay,20 suggesting that impaired wound NO synthesis is a key factor for the delayed healing. However, the underlying mechanisms responsible for NO deficiency–induced wound healing impairment remain to be delineated. In the present study, we tested the hypothesis that hyperglycemia impairs cutaneous NO function and wound repair through O2− generation, which may be reversed by gene therapy of eNOS or manganese superoxide dismutase (MnSOD) in type 1 diabetic mice. Our results indicate (1) that cutaneous O2− levels were significantly increased in chemical substance streptozotocin-induced and autosomal dominant mutation in Ins2 gene–induced Ins2Akita diabetic mice, and (2) that decreased eNOS protein and activity contribute to wound healing delay and in vivo gene therapy of eNOS or MnSOD promotes the healing of full-thickness excisional wounds in streptozotocin-induced diabetic mice.

Methods

Induction of Type 1 Diabetes
This study was approved by the animal research committees of Michigan State University and Chinese Third Military Medical University. Male C57/B6 mice (Jackson Laboratories, Bar Harbor, Maine) aged 8 to 12 weeks were injected intraperitoneally with streptozotocin (Sigma Chemical Co) in dissolved sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5, 45 mg/kg). Streptozotocin or citrate buffer (control) was administered for 5 consecutive days during the first week of the study.21 Blood was collected from the dorsal vein of the mouse hind foot with the use of Microvette blood collection tubes (Microvette CB 300 LH, Sarstedt). Whole-blood glucose levels were measured by a glucose analyzer (2300 Stat Plus analyzer, YSI Incorporated). Mice with a blood glucose level >280 mg/dL were considered diabetic and used for wound experiments.21 Ins2−/− diabetic mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Ins2−/− diabetic mouse is an autosomal dominant mutation in Ins2 gene and is well-known animal model of type 1 diabetes.

Excisional Wound Model
Ten weeks after streptozotocin or citrate buffer injection, mice were anesthetized with ketamine (100 mg/kg IP; Sigma Chemical Co) and xylazine (10 mg/kg IP; Sigma Chemical Co), and the dorsum was clipped free of hair. A full-thickness excisional wound (1.5 × 1.5 cm), including the panniculus carnosus, was created with fine scissors.18 Wounds were dressed with a Bioclusive transparent dressing (Johnson & Johnson), and wound closure rates were measured by tracing the wound area every other day onto the Bioclusive dressing. The tracings were digitized, and the areas were calculated in blinded fashion with the use of a computerized algorithm (Sigma Scan; Jandel Scientific).

Adenoviral Vector–Mediated Gene Therapy
Replication-incompetent adenoviral vectors, driven by the cytomegalovirus immediate-early promoter encoding a cDNA sequence for MnSOD, eNOS, dominant-negative HA-tagged N17Rac1, which inhibits Rac1, the small GTPase component of NADPH oxidase (N17Rac1, a kind gift from Dr John Engelhardt, University of Iowa, Iowa City), or β-galactosidase (β-gal) reporter gene, were propagated, purified, and titrated as previously described.22,23 The prepared vectors were stored at −80°C in 0.01 mol/L Tris, 0.01 mol/L MgCl2, and 10% glycerol before use. In vivo gene transfer was performed as described.18 Briefly, after the wound was created on the dorsum of mice, gene transfer of eNOS, MnSOD, or β-gal was immediately performed in some animals by placing 200 μL of viral solution (108 plaque-forming units [pfu]/mL) directly onto the wound surface for 30 minutes. The solution was then removed with sterile gauze, and the wounds were covered with a Bioclusive dressing to maintain sterility. For ex vivo gene transfer, skin tissues from Ins2−/− diabetic mice were randomly exposed to eNOS, MnSOD (105 pfu/mL), dominant-negative HA-tagged N17Rac1 (N17Rac1), or β-gal (105 pfu/mL) in Eagle’s minimal essential medium (EMEM) for 4 hours followed by incubation in fresh EMEM for 20 hours, or skin tissues from normal mice were randomly exposed to either N17Rac1 or β-gal (105 pfu/mL) in EMEM for 4 hours followed by incubation in fresh EMEM with 300 mg/dL glucose for 20 hours. Some skin tissues were incubated in EMEM for 24 hours without gene transfer. All skin tissues were used for O2− measurement.

Immunoblotting of eNOS
Western blot analysis for eNOS protein was performed as described.23 Briefly, skin tissues were homogenized in lysis buffer (0.5 mol/L Tris HCl [pH 6.8], 10% SDS, 10% glycerol) with protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin (all from Sigma Chemical Co). Homogenates were centrifuged (11 000g) for 10 minutes, 4°C, and the protein supernatants were measured. Equal amounts of protein (6.8 μg) were separated on SDS-polyacrylamide gels (7.5%) and electrotransferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) and blotted with the primary antibody against eNOS (1:2500; BD Transduction Laboratories). Human endothelial lysate (BD Transduction Laboratories) was used as a positive control for eNOS. The secondary antibody used was mouse anti-goat antibody (1:2000; BD Transduction Laboratories). Blots were developed with the use of Supersignal West Pico chemiluminescent substrate (Pierce) and reprobed with actin (Santa Cruz Biotechnology, Inc.). Molecular band intensity was determined by densitometry (Bio-Rad image software).

Radioimmunoassay of NO Activity
Skin tissues were homogenized and centrifuged, and supernatants were subjected to NO activity assay with a commercial NO radioimmunoassay (RIA) kit (Calbiochem). Aliquots of supernatants were incubated with [1H]arginine (10 μmol/L final arginine, 62 μmolar, Amersham) in the presence of 1 mmol/L NADPH, 3 μmol/L tetrahydrobiopterin, 600 μmol/L CaCl2, 1 μmol/L flavin-adenine dinucleotide, and 1 μmol/L flavin mononucleotide in a final volume of 50 μL for 60 minutes at room temperature.24 The reaction was quenched with 400 μL of stop buffer (50 mmol/L HEPES and 5 mmol/L EDTA, pH 5.5). Experiments were also performed in the presence of either EGTA (5.0 mmol/L) or Nω-l-arginine methyl ester (L-NAME) (1 mM/L).24 After resin was added, the reaction mixtures were transferred to spin cups and centrifuged. 1-[1H]Citrulline content in eluate was determined by liquid scintillation counting. Samples of buffer containing 1-[1H]arginine in the absence of skin tissue served as background counts, which were subtracted from all measurements. Constitutive NOS (eNOS and neuronal NOS) activity was determined by subtracting total counts from both L-NAME– and calcium chelator EGTA–blocked counts and normalized for protein content (measured by the Bradford assay [Bio-Rad]).

Nitrite Measurement
When the wound was completely healed, the wound tissue was cut into small pieces that were used to assay the nitrite level with Griess reagents (Sigma Chemical Co). After incubation with EMEM in a CO2 incubator for 24 hours, the wound tissue was weighed, and the NO stable metabolite nitrite concentration in EMEM was determined.

SOD Activity Measurement
Skin tissues were homogenized in cold buffer (20 mmol/L HEPES, 1 mmol/L EGTA, 210 mmol/L mannitol, and 70 mmol/L sucrose), and homogenates were centrifuged at 13 000g for 15 minutes at 4°C.25,26 The resulting supernatant that contained cytosolic SOD was subjected to an SOD activity assay with a commercial SOD kit.
(Cayman Chemical). Briefly, the addition of 1 mmol/L potassium cyanide was used to inhibit both Cu/Zn-SOD and extracellular SOD, resulting in the detection of only MnSOD activity. Hypoxanthine/xanthine oxidase was used to generate O$_2^-$, which was detected by tetrazolium salt through reading the absorbance at 450 nm.

**Superoxide Measurement**

Cutaneous O$_2^-$ levels were quantified by lucigenin-enhanced chemiluminescence as described. Skin tissues clipped free of hair were cut into small pieces (2×2 mm) and placed in polypropylene tubes containing 5 mmol/L lucigenin in 1 mL modified Krebs’ solution. Tubes were read in a luminometer (TD-20/20, Turner Designs). The luminometer reports relative light units emitted, which were integrated over 5 minutes. The superoxide-sensitive fluorescent dye dihydroethidium was used to evaluate in situ production of O$_2^-$, which was detected by tetrazolium salt through reading the absorbance at 450 nm.

**Statistical Analysis**

Data are presented as mean±SEM. Experimental means were subjected to unpaired Student t test and 1-way ANOVA with Newman-Keuls multiple comparison test. A probability value of <0.05 was considered statistically significant.

**Results**

**Streptozotocin-Induced and Ins2Akita Diabetic Mice**

To test the role of hyperglycemia on wound repair without the influence of insulin, a 5-day low-dose streptozotocin injection regimen was used to ensure 13 weeks of sustained hyperglycemia, which was required to impair wound healing. Under such a regimen, streptozotocin-treated mice maintained hyperglycemia and experienced body weight loss throughout the study (Figure 1). The glucose levels were significantly elevated (308±30 versus 98±10 mg/dL; $P<0.05$), and body weights were markedly decreased (24.1±0.67 versus 29.5±0.30 g; $P<0.05$) in 16-week-old Ins2Akita diabetic mice compared with control mice.

**Cutaneous Gene Therapy of eNOS or MnSOD on Wound Healing in Diabetic Mice**

The rate of wound closure in streptozotocin-induced diabetic mice to a 100% closure point was delayed by 23.6% compared with that of the control mice. In vivo gene transfer of eNOS or MnSOD accelerated the closure rates by an average of 15.7% and 13.8% to a 100% closure point, respectively, compared with the nontransduced diabetic mice or mice transduced with β-gal reporter gene (Figure 2).

**Impaired Wound-Induced Endogenous eNOS Expression and NOS Activity in Diabetic Mice**

Endogenous eNOS protein levels in skin tissues were not significantly different between normal and diabetic mice (Table and Figure 3A through 3C). However, constitutive NOS activity was markedly decreased in diabetic mice compared with normal mice (Figure 4A). Specially, there was significant impairment of wound-induced endogenous eNOS expression during the wound healing process in diabetic mice compared with normal mice (Table and Figure 3A through 3C). Similarly, wound-induced activation of cutaneous NOS was absent in diabetic mice (Figure 4A).

![Figure 1. Whole-blood glucose concentration (A) and body weight (B) in streptozotocin (STZ)-induced diabetic mice (45 mg/kg, 5 days) and control mice (citrate buffer). Values are mean±SEM; n=15 for STZ mice and n=6 for control mice. *$P<0.05$ vs control.](http://circ.ahajournals.org/doi/fig/10.1161/CIRCULATIONAHA.104.526021)

![Figure 2. Effects of cutaneous gene therapy of eNOS or MnSOD on wound healing in diabetic mice. Ten weeks after streptozotocin (STZ) treatment, a full-thickness excisional wound (1.5×1.5 cm) was made, and eNOS or MnSOD (3×10$^{10}$ pfu in 0.2 mL of solution) was placed onto the wound for 30 minutes. The rate of wound closure was monitored every other day by tracing the wound area. Values are mean±SEM; n=3 to 5. *$P<0.05$ vs control; #$P<0.05$ vs STZ.](http://circ.ahajournals.org/doi/fig/10.1161/CIRCULATIONAHA.104.526021)
Gene Therapy of eNOS or MnSOD on Cutaneous eNOS Protein Expression, NOS Activity, and NO Levels in Diabetic Mice

In vivo gene transfer of eNOS or MnSOD onto the wounds of diabetic mice resulted in a significant increase of eNOS protein during the wound healing process on days 1, 3, 5, 7, 10, and 14 after wounding compared with nontransduced diabetic mice, whereas gene transfer of β-gal had no such effect (Table and Figure 3B through 3E). Similarly, constitutive NOS activity was significantly increased on days 1, 7, and 14 after wounding in eNOS- or MnSOD-transduced diabetic mice compared with nontransduced or β-gal–transduced diabetic mice (Figure 4A). Cutaneous NO levels in eNOS- or MnSOD-transduced diabetic mice were increased by 177.3% and 40.5%, respectively, an effect that was inhibited by NOS inhibitor L-NAME (10⁻⁴ mol/L) (Figure 4B).

<table>
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<tr>
<th>Days After Wound</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>14</th>
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<tr>
<td>Control</td>
<td>0.682±0.126</td>
<td>0.621±0.081*</td>
<td>0.792±0.169†</td>
<td>1.225±0.07‡</td>
<td>1.104±0.089§</td>
<td>1.027±0.052∥</td>
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<tr>
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<td>0.491±0.054</td>
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<td>STZ/eNOS</td>
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<td>1.491±0.112*</td>
<td>1.142±0.122†</td>
<td>1.145±0.121‡</td>
<td>1.072±0.119§</td>
<td>0.965±0.049</td>
<td>0.928±0.131¶</td>
</tr>
<tr>
<td>STZ/MnSOD</td>
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<td>1.297±0.205†</td>
<td>1.167±0.111‡</td>
<td>1.384±0.094§</td>
<td>1.490±0.137∥</td>
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</tr>
<tr>
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<td>0.561±0.151</td>
<td>0.646±0.074</td>
<td>0.726±0.077</td>
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Values are mean±SEM; n=3 to 5. STZ indicates streptozotocin.
*P<0.05 vs STZ 1-day group.
†P<0.05 vs STZ 3-day group.
‡P<0.05 vs STZ 5-day group.
§P<0.05 vs STZ 7-day group.
∥P<0.05 vs STZ 10-day group.
¶P<0.05 vs STZ 14-day group.

Figure 3. Time course of eNOS expression after cutaneous gene therapy of eNOS or MnSOD in diabetic mice. eNOS expression was determined by Western blot analysis on days 0, 1, 3, 5, 7, 10, and 14 after wounding. Gene therapy of eNOS, MnSOD, or β-gal was performed immediately after wounding (A to F). Values are mean±SEM; n=3 to 5. *P<0.05 vs day 0; #P<0.05 vs day 1. STZ indicates streptozotocin.
Gene Therapy of MnSOD on Cutaneous MnSOD Activity in Diabetic Mice

In vivo gene transfer of MnSOD onto the wounds in diabetic mice resulted in a significant increase of cutaneous cytosolic MnSOD activity during the wound healing process on days 1, 7, and 14 after wounding compared with nontransduced diabetic mice, whereas gene transfer of β-gal had no such effect (Figure 4C).

Gene Therapy of eNOS or MnSOD on Cutaneous Superoxide Levels in Diabetic Mice

In streptozotocin-induced and Ins2Akita diabetic mice, cutaneous $O_2^-$ levels were markedly higher than that of the control mice. In vivo gene transfer of eNOS or MnSOD impeded $O_2^-$ levels in streptozotocin-induced diabetic mice when measured on wound closure (Figure 5A). Ex vivo gene transfer of eNOS or MnSOD also reduced the $O_2^-$ levels in Ins2Akita diabetic mice (Figure 5C).

Inhibition of PKC and NADPH Oxidase Reduces High Glucose–Induced Cutaneous $O_2^-$

The increased cutaneous $O_2^-$ levels in streptozotocin-induced and Ins2Akita diabetic mice were significantly decreased after the skin tissues were treated for 30 minutes in vitro with protein kinase C inhibitor chelerythrine ($3 \times 10^{-6}$ mol/L) or NADPH oxidase inhibitor apocynin ($10^{-4}$ mol/L) or diphenyleneiodonium ($10^{-4}$ mol/L) (Figure 5B to 5C). In contrast, inhibition of xanthine oxidase by allopurinol ($10^{-4}$ mol/L) or NOS by L-NAME ($10^{-4}$ mol/L) failed to produce the same effect in streptozotocin-induced diabetic mice (Figure 5B). Ex vivo gene transfer of dominant-negative HA-tagged N17Rac1 significantly inhibited cutaneous $O_2^-$ in Ins2Akita diabetic mice, whereas gene transfer of β-gal failed to produce such an effect (Figure 5C). Similarly, in vitro glucose treatment of skin tissues from normal mice increased $O_2^-$ levels in a concentration-dependent manner (Figure 6A), an effect that was blunted by chelerythrine, apocynin, or diphenyleneiodonium ($10^{-4}$ mol/L) but not allopurinol or L-NAME (Figure 6B). Ex vivo gene transfer of dominant-negative HA-tagged N17Rac1 also significantly inhibited cutaneous $O_2^-$ induced by 300 mg/dL glucose (Figure 6B to 6C).

Discussion

The present study demonstrates, for the first time, (1) that high glucose levels induce cutaneous superoxide via NADPH oxidase and protein kinase C, resulting in impaired NO function and delayed wound healing, and (2) that cutaneous gene therapy of eNOS or MnSOD increases eNOS expression and function, resulting in accelerated wound healing in type 1 diabetic mice.

Reduced Cutaneous NO Bioavailability Contributes to Diabetic Wound Healing Impairment

There is growing evidence that NO plays a pivotal role in normal wound healing. All 3 NOS isoforms, including eNOS, iNOS, and neuronal NOS (nNOS), are expressed in skin tissues. Expression of eNOS can be detected in keratinocytes of the basal epidermal layer, dermal fibroblasts, endothelial capillaries, and eccrine glands. NO serves as an autocrine and paracrine mediator in maintaining normal skin homoeostasis. It promotes processes central to wound healing, including angiogenesis and migration and proliferation of fibroblasts, epithelial cells, endothelial cells, and keratinocytes.
However, the direct effects of NO on wound repair under pathological conditions remain unknown. Our results demonstrate that although endogenous eNOS protein level in skin tissues was not different between normal and diabetic mice before wounding, its expression was significantly impaired during the wound healing process only in diabetic mice. More importantly, constitutive NOS activity was markedly decreased in diabetic mice compared with normal mice, with a concomitant reduction of the stable NO metabolite nitrite. These findings suggest that impairment of wound-induced endogenous eNOS expression and NOS activity is responsible for reduced cutaneous NO bioavailability in type 1 diabetic mice. These results are also in agreement with previous studies showing that impaired wound healing was paralleled by decreased wound eNOS expression and that treatment with insulin and L-arginine partially restored NO production and improved wound healing in diabetic animals.

To ascertain that impairment of cutaneous NO function contributes to delayed wound healing, we investigated the direct effect of gene therapy of eNOS or MnSOD on wound healing in type 1 diabetic mice. The results show that gene therapy of eNOS or MnSOD onto the wounds in diabetic mice significantly accelerated wound healing. The observed therapeutic effect is most likely due to enhanced wound NO bioavailability because cutaneous eNOS protein expression and constitutive NOS activity were both significantly increased in eNOS- or MnSOD-transduced diabetic mice compared with nontransduced or β-gal–transduced diabetic mice. Furthermore, the augmented endogenous eNOS expression observed in normal mice during wound healing was absent in diabetic mice, a process that was reversed by eNOS or MnSOD gene therapy. Because wounding per se significantly induced cutaneous eNOS expression in normal mice, as shown in this study, it would be difficult to distinguish endogenous and exogenous eNOS expression in transduced normal mice. Hence, a comparison of eNOS expression in transduced normal mice and diabetic mice may not yield such desired information. Collectively, our data indicate that reduced eNOS protein and activity contribute to delayed wound healing and demonstrate, for the first time, that increased local NO bioavailability via cutaneous gene therapy is an efficacious means to improve wound healing in diabetic mice.

Although a central role for protein-type growth factors and mitogens during wound repair has been well known for years, the application of these factors in the treatment of wound healing has not provided reliable treatments in clinical studies. One possible explanation for the failure of protein-type mitogens to markedly accelerate closure of chronic wounds may be tremendously elevated protease activities in the fluids of chronic wounds. These findings suggest that the molecular environment of chronic wounds may impair the ability of exogenously applied growth factors to stimulate healing. The results of the present study suggest that NO might represent a novel target molecule that circumvents the difficulties that arise because of proteolytic cleavage of therapeutically applied growth factors in chronic wound situations. Because NO is a short-lived gas molecule, maintenance of an effective level of NO at a wound site is difficult. Our results demonstrate proof of the principle that cutaneous gene therapy of eNOS or MnSOD may be an efficacious means to achieve a
sustained local NO level at the wound site. However, future studies are warranted to determine the optimal gene dose regimens.

**Increased Cutaneous \( \text{O}_2^- \) Contributes to Decreased NO Bioavailability**

Although impaired NO function contributes to delayed wound healing in diabetes, the mechanisms underlying cutaneous NO dysfunction are not clear. Causative factors for hyperglycemia-induced tissue damage include activation of the polyol pathway, nonenzymatic glycation and protein kinase C pathways, and increased hexosamine pathway flux. However, there was no apparent common element linking these known mechanisms. Recent studies suggest that these different mechanisms may be linked to a single cellular process: an overproduction of \( \text{O}_2^- \) induced by sustained hyperglycemia. Sustained hyperglycemia is known to increase vascular \( \text{O}_2^- \) levels, which results in cardiovascular dysfunction. \( \text{O}_2^- \) produced in the vasculature rapidly inactivates NO and reduces its bioactivity in diabetic blood vessels. Independent strategies aimed at reducing \( \text{O}_2^- \) formation have been shown to prevent high glucose–induced protein kinase C activation, formation of advanced glycation end products, sorbitol accumulation, and nuclear factor-\( \kappa \)B activation, resulting in the improvement of endothelium-dependent NO-mediated vasodilatation as well as wound healing. These studies suggest that the increase in \( \text{O}_2^- \) levels is a key factor for NO dysfunction in diabetes. However, the effects of hyperglycemia on cutaneous \( \text{O}_2^- \) and NO levels in diabetes remain unknown.

The results of the present study demonstrate that in vitro glucose treatment of skin tissues from normal mice in-
creased $O_2^-$ levels in a concentration-dependent manner and that there is a marked increase of cutaneous $O_2^-$ levels in chemical substance streptozotocin-induced type 1 diabetic mice and autosomal dominant mutation in Ins2 gene–induced type 1 diabetic mice. Gene therapy of MnSOD significantly elevated MnSOD activity, resulting in decreased $O_2^-$ and increased NO levels in streptozotocin-induced type 1 diabetic mice. These findings are consistent with a previous study showing that inhibition of lipid peroxidation restored wound healing to nearly normal levels in diabetes-impaired wounds. Because the vector titer used for MnSOD gene therapy was exactly the same as for eNOS and β-gal, the profile of MnSOD transgene expression should also be similar. The augmented MnSOD activity 14 days after MnSOD transduction supports this stipulation. The increased cutaneous NO levels may be a combined result of reduced $O_2^-$ inactivation of NO and augmented NO protein expression and activity after MnSOD gene therapy. Although the mechanism of enhanced NO protein expression and activity is unclear, it may be related to the formation of hydrogen peroxide after MnSOD gene therapy. This speculation is based on previous experimental observations demonstrating that hydrogen peroxide is an effective stimulus of eNOS expression in endothelial cells of animals and humans.

NADPH Oxidase and Protein Kinase C Are Key Sources for Cutaneous $O_2^-$ Production

In diabetic vasculature, the main enzymatic sources for $O_2^-$ production include NADPH oxidase, xanthine oxidase, mitochondrial oxidase, and uncoupled eNOS. However, the humoral factors responsible for $O_2^-$ production in diabetic wounds are unknown. NADPH oxidase is expressed in endothelial cells, fibroblasts, neutrophils, and phagocytes, and it is composed of multiple subunits in which the small G protein Rac1 is required for its activation. Apocynin is a selective NADPH oxidase inhibitor that prevents the binding of the cytosolic subunits to the membrane-bound subunits, thus preventing oxidase activation and subsequent $O_2^-$ production. Overexpression of dominant-negative Rac1 has been shown to inhibit Rac1 activity, resulting in NADPH oxidase inactivation and a lack of $O_2^-$ generation. In the present study we observed that both apocynin, a selective inhibitor of NADPH oxidase, and chelerythrine, a selective inhibitor of protein kinase C, suppressed cutaneous $O_2^-$ formation induced by high glucose in both normal mice and diabetic mice. Moreover, gene transfer of dominant-negative HA–tagged N17Rac1 also significantly inhibited glucose-induced cutaneous $O_2^-$ production. In contrast, neither NOS inhibitor L-NAME nor xanthine oxidase inhibitor allopurinol produced similar effects. Because cutaneous gene therapy of eNOS increased local NO and reduced $O_2^-$ levels, these results also suggest that eNOS itself does not play a significant role in glucose-induced cutaneous $O_2^-$ generation.

MnSOD has been shown to be mainly localized to mitochondria and plays an important role in protecting against oxidative stress–induced tissue damage. In bovine endothelial cells, disruption of mitochondrial $O_2^-$ production was achieved with several different approaches, including the following: (1) treatment with carbonyl cyanide m-chlorophenylhydrazone, a small-molecule uncoupler of mitochondrial oxidative phosphorylation; (2) overexpression of uncoupling protein-1, a protein uncoupler; or (3) overexpression of MnSOD. Each of these approaches blocked the hyperglycemia-induced increase in $O_2^-$ production. Consequently, the hyperglycemia-induced effects on nuclear factor-κB, protein kinase C, advanced glycation end products, and sorbitol were also suppressed. The results of the present study demonstrated that in vivo or ex vivo MnSOD gene transfer resulted in increased MnSOD activity and increased NO levels and wound repair in streptozotocin-induced diabetic mice.

Oxidants and Antioxidants and Wound Healing

Results of a recent study showed that increased oxidants accelerated cutaneous wound healing in normal mice and induced vascular endothelial growth factor expression in human keratinocytes. Low concentrations of reactive oxygen species (ROS), including $O_2^-$, hydrogen peroxide, and hydroxyl radicals, may serve as signaling messengers and regulate gene expression processes. Protein-type growth factors such as platelet-derived growth factor and transforming growth factor regulate cell growth through ROS-dependent signal pathways. These results suggest that low concentrations of ROS may be necessary for maintaining normal cell function. However, it is well established that $O_2^-$ overproduction leads to cell injury under pathological states, including diabetes. Consistent with this notion, previous studies have shown that antioxidants accelerated diabetic wound healing. On the basis of these studies and our own findings, it is reasonable to speculate that high glucose–induced cutaneous $O_2^-$ increase contributes, at least in part, to impaired NO function in diabetic wounds, and antioxidant strategies may be beneficial for improving diabetic wound healing.

In summary, the present study demonstrates, for the first time, that cutaneous $O_2^-$ levels are markedly increased in streptozotocin-induced and autosomal dominant mutation in Ins2 gene–induced type 1 diabetic mice and in skin
tissues from normal mice treated with high glucose in vitro, and (2) that cutaneous gene therapy of eNOS or MnSOD promotes wound healing in streptozotocin-induced type 1 diabetic mice, an effect attributable to decreasing O$_2^-$ levels induced by NADPH oxidase, protein kinase C, and mitochondria. Gene therapy strategies aimed at reducing O$_2^-$-induced wound healing impairment may be an effective means of improving wound healing delay in diabetes.

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