An essential role of NRF2 in diabetic wound healing

Running title: NRF2 improves diabetic wound healing

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ABSTRACT

The high mortality and disability of diabetic non-healing skin ulcers create an urgent need for the development of more efficacious strategies targeting diabetic wound healing. In the current study, using human clinical specimens we show that perilesional skin tissues from diabetic patients are under more severe oxidative stress and display higher activation of the NRF2-mediated antioxidant response than perilesional skin tissues from normoglycemic patients. In an STZ-induced diabetes mouse model, $Nrf2^{-/-}$ mice have delayed wound closure rates compared to $Nrf2^{+/+}$ mice, which is, at least partially, due to greater oxidative DNA damage, low TGF-β1 and high MMP9 expression, and increased apoptosis. More importantly, pharmacological activation of the NRF2 pathway significantly improves diabetic wound healing. In vitro experiments in HaCaT cells confirm that NRF2 contributes to wound healing by alleviating oxidative stress, increasing proliferation and migration, decreasing apoptosis, and increasing the expression of TGF-β1 and lowering MMP9 under high glucose conditions. This study indicates an essential role for NRF2 in diabetic wound healing and the therapeutic benefits of activating NRF2 in this disease, laying the foundation for future clinical trials using NRF2 activators in treating diabetic skin ulcers.
Chronic non-healing skin ulcers are a major cause of disability and mortality in the diabetic population (1; 2). Cutaneous wound healing is a complex process comprising coagulation, inflammation, migration-proliferation, and remodeling (3; 4). During the first stages many growth factors, including transforming growth factor (TGF), are released (5). TGF-β1 plays a crucial role in the recruitment of inflammatory cells as well as in the synthesis and deposition of extracellular matrix (ECM) (6). The migration-proliferation and remodeling stages, which occur several weeks after wounding and can last up to several months, involve ECM deposition, angiogenesis, migration, proliferation, contraction, and tissue remodeling. Keratinocytes produce various factors which regulate angiogenesis, granulation tissue formation, ECM remodeling, re-epithelialization, and proliferation of keratinocytes and fibroblasts (7-10). Proliferation is important since cell migration alone is insufficient to close large and full-thickness wounds (11). Keratinocyte migration is facilitated by ECM degradation by matrix metalloproteinases (MMPs), but excessive MMP activity delays wound healing (12). Diabetic wound healing differs from the normal process due to intrinsic pathophysiological abnormalities (reduced blood supply, impaired wound contraction and matrix turnover) and extrinsic factors (infection and repeated trauma) that lead to delayed and aberrant wound healing processes (3; 13). Furthermore, many studies have identified that chronic oxidative stress associates with the progression of diabetic complications and impaired wound healing (14; 15).

The transcription factor NRF2 (nuclear factor-E2-related factor 2) regulates the adaptive response to exogenous and endogenous oxidative stresses (16; 17), as well as cell migration, proliferation, apoptosis, and differentiation (18-22). NRF2 is considered an attractive druggable target for cancer, neurodegenerative diseases, liver cirrhosis, diabetes, and wound healing (17; 23-26), and we have previously demonstrated the protective role of NRF2 and the potential
therapeutic effect of NRF2 activators in a diabetic nephropathy animal model (27; 28). The diet-derived chemopreventive compounds sulforaphane (SF) and cinnamaldehyde (CA) are two well-characterized NRF2 activators with great potential to be used therapeutically due to their lack of toxicity at the doses required to activate NRF2 (27; 29; 30).

The present study aimed to explore the role of NRF2 in diabetic wound healing. Perilesional skin tissue samples from diabetes ulcer patients and normoglycemic trauma patients were used to detect oxidative stress levels and NRF2 activation. In a diabetes mouse model, NRF2 was pharmacologically activated to decrease oxidative stress and accelerate wound closure of diabetic mice. Finally, in vitro experiments were performed to elucidate some of the mechanisms by which NRF2 activation promotes diabetic wound healing. This study provides convincing experimental evidence that the NRF2 signaling pathway contributes to the wound healing process, suggesting NRF2 activators may be used to treat skin ulcers in diabetic patients.

RESEARCH DESIGN AND METHODS

Chemicals, antibodies and cell culture. CA, streptozotocin (STZ), and 2′,7′-dichlorofluorescein diacetate (DCF) were purchased from Sigma (St. Louis, MO). L-SF was from LKT laboratories (St. Paul, MN). Primary antibodies against Ki67, NRF2, MMP9, HO-1, AKR1C1, TGF-β1, and Actin, as well as horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-8-dihydro-2-deoxyguanosine (8-oxo-dG) antibody was from Trevigen (Gaithersburg, MD).

Human immortalized keratinocytes (HaCaT) were obtained from Dr. Tim Bowden, from the Arizona Cancer Center. Cells were grown in DMEM (Cellgro, Manassas, VA) containing low glucose (LG, 5.5 mmol/L) and 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. For the experiments, cells were starved in serum-free LG for 24 h, then either maintained in LG or
switched to high glucose (HG, 25 mmol/L) DMEM for 2 days. For the treatments, the cells were
dosed with 5 µmol/L SF or 20 µmol/L CA.

**siRNA transfections.** HaCaT cells were transfected with either a control small interfering RNA
(Con-siRNA, #1027281, Qiagen, Valencia, CA) or an *NRF2*-specific siRNA (#S100657937)
using the HiPerfect transfection reagent (Qiagen) according to the manufacturer’s instructions.
Briefly, cells were maintained in LG or HG for 2 days and then transfected with siRNA for
migration, proliferation, and apoptosis experiments. For ROS detection, LG cells were
transfected with siRNA and 24 h later switched to HG and treated as indicated.

**Human skin tissue samples.** Perilesional skin tissue samples were obtained from ulcers of 12
patients with diabetes mellitus (Supplementary Table 1) and from 11 normoglycemic patients
who needed debridement due to trauma. All normoglycemic patients had no medical history of
diabetes (fasting blood glucose and glycosylated hemoglobin in the normal range) and did not
suffer from general infection, cardiovascular, or renal diseases. All tissue samples included a 1
cm margin surrounding the wound. Permission to use the fixed tissue sections for research
purposes was obtained and approved by the Ethics Committee of the Xinqiao Hospital, Third
Military Medical University of People’s Liberation Army, China, and a written consent form was
obtained from all patients.

**Diabetes mouse model and treatments.** *Nrf2*+/+ and *Nrf2*−/− C57BL/6 mice were described
previously (28) and were housed and handled in accordance with the University of Arizona
Institutional Animal Care policies. The STZ-induced diabetic model was previously described
(27) and only 8-week-old male mice were included. Briefly, three weeks after STZ injections, fasting glucose levels (FGL, 4 h fast) were measured and all mice had FGL ≥ 250 mg/dL, and thus were considered diabetic and included in the study (see Fig. 2A for a detailed timeline). Non-diabetic control mice received sodium citrate buffer (pH 4.5) injections instead. Mice (n=8/group) were randomly allocated to the indicated treatment groups and received corn oil (vehicle control), 12.5 mg/kg SF, or 50 mg/kg CA intraperitoneally every two days until skin tissues were harvested (27; 29). One week after the treatments, the mice were anesthetized, their backs were shaved and cleaned. Two wounds were made with a sterile 6 mm skin biopsy punch (Healthlink, Jacksonville, FL) and covered with 3M Tegaderm pads (St. Paul, MN). The wounds were photographed using an in vivo imaging system and infrared thermography (IRISYS, San Diego, CA) for 13 days. Gross wound closure was quantified with ImageJ, and wound healing was expressed as the percentage of the original wound area that had healed. Tissues were collected using an 8 mm skin biopsy punch; one half was fixed in 10% buffered formalin and embedded in paraffin, the other half was used for protein extraction. Mice that were too weak or developed wound complications were excluded from the analysis.

**Immunohistochemistry (IHC), immunoblotting, TGF-β1 immunoassay, and gelatin zymography.** Human and mouse skin tissue morphology was assessed by hematoxylin and eosin (H&E) staining (Vector, Burlingame, CA). IHC and oxidative DNA damage detection (8-oxo-dG) were performed as described previously (27); staining was performed using the Envision system HRP-DAB kit (DAKO, Carpinteria, CA) according to the manufacturer’s instructions.

For protein detection, tissue and cell lysates were prepared as previously described (27). Total lysates were resolved by SDS-PAGE and immunoblotting with the indicated antibodies. To
detect the secreted proteins TGF-β1 and MMP9, cells were incubated 24 h in serum-free DMEM before harvesting the medium. TGF-β1 was measured using the Quantikine human TGF-β1 immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The absorbance at 450 nm was measured using a Synergy 2 microplate reader (BioTek, Winooski, VT) and the amount of TGF-β1 was calculated by standard curve. MMP9 activity was evaluated by gelatin zymography as described elsewhere (31). Coomassie blue-stained gels were analyzed with ImageJ.

**In vitro wounding assay.** The wound healing assay was performed as described elsewhere (32). Briefly, UV-sterilized PDMS blocks (1 mm × 2 cm) were placed transversally onto 35 mm glass bottom dishes. HaCaT cells were seeded at a density of 100,000 cells/mL and after 48 h the slab was removed to allow migration for 72 h. The cells were treated with 5 µmol/L SF or 20 µmol/L CA according to our previous studies (33; 34)at the time of seeding and every 24 h until the end of this experiment. For siRNA experiments, the cells were transfected at the time of seeding.

**Reactive oxygen species (ROS) measurement, cell proliferation and apoptosis.** ROS were measured in HaCaT cells using DCF. Briefly, the cells were seeded in LG or HG and treated with 5 µmol/L SF or 20 µmol/L CA every 24 h (33; 34). After 48 h, the cells were switched into fresh medium containing 10 µg/mL DCF, incubated 30 min, and fluorescence intensity was measured by flow cytometry. The rate of cell proliferation was measured by detection of Ki67 using indirect immunofluorescence as described previously (35), and with the xCELLigence system (Roche, Indianapolis, IN). For this, 8000 HaCaT cells/well were seeded in LG or HG, with or without NRF2 activators, and cell growth was monitored for 72 h. The In Situ Cell Death
Detection Kit (Roche) (TUNEL) was used to detect apoptosis according to the manufacturer’s instructions. Fluorescent images were taken using a Zeiss Observer.Z1 microscope with the Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO).

**Statistical analysis.** Results are expressed as means ± SEM. Statistical tests were performed using GraphPad Prism 6.0 (La Jolla, CA). *In vitro* experiments were done in triplicate. One-way ANOVA with Tukey’s post hoc test was applied to compare the means of three or more groups. Unpaired, two-sided Student’s t tests were used to compare the means of two groups. $P < 0.05$ was considered to be significant.

**RESULTS**

**Perilesional skin tissues of diabetic patients are under severe oxidative stress that activates the NRF2-mediated antioxidant response.** Perilesional skin tissues were collected from normoglycemic and diabetic patients for pathological (H&E) and immunohistochemical analyses (IHC). Diabetic skin had more inflammatory cells infiltration, edema, and less granulation tissue formation than normoglycemic skin, indicating an impaired wound healing process in diabetes (Fig. 1A and B). Normoglycemic tissues showed moderate oxidative DNA damage (8-oxo-dG staining) whereas in diabetic tissues it was stronger, indicating that skin adjacent to wounds is under oxidative stress and diabetic wound tissue has greater oxidative DNA damage (Fig. 1I and J). Additionally, TUNEL analysis identified a marked induction of apoptosis in diabetic skin, and less apoptosis was present in normoglycemic skin (Fig. 1K and L). As a consequence of high oxidative stress, NRF2 and its downstream genes (HO-1 and NQO1) were greatly activated in the epidermal layer of diabetic wounds skin (Fig. 1C-H). These results suggest that skin tissues
of diabetic patients undergo severe oxidative damage which causes apoptosis and compensatory NRF2 pathway activation.

**SF and CA activate NRF2 in skin tissues of STZ mice.** Based on the observation that the wounds of diabetic patients have increased oxidative stress, which in turn activates the NRF2 antioxidant response, wound tissues of STZ-induced diabetic mice were analyzed. Two wounds were made in the backs of Nrf2+/+ (n=5) and Nrf2−/− (n=6) C57BL/6 mice and were photographed to compare and quantify wound closure (Supplementary Fig. 1A). Wound healing was slower in diabetic Nrf2−/− than in diabetic Nrf2+/+ mice at days 3, 7, and 14 after surgery (Supplementary Fig. 1B and C). As expected, higher 8-oxo-dG and more apoptotic cells was detected in diabetic Nrf2−/− mouse skin compared to diabetic Nrf2+/+ mouse skin (Supplementary Fig. 1D). Moreover, diabetic Nrf2−/− mouse skin had lower TGF-β1 and higher MMP9 expression than Nrf2+/+ mouse skin (Supplementary Fig. 1D). All these results indicate that diabetic Nrf2−/− mice suffered delayed wound healing due to lack of NRF2-mediated compensatory protection (Supplementary Fig. 1D), and suggest that NRF2 contributes to diabetic wound healing. Therefore, it was hypothesized that pharmacological activation of NRF2 starting before the wounding and sustained throughout the whole healing process might improve diabetic wound healing.

To explore the feasibility of pharmacologic activation of NRF2 to facilitate diabetic wound healing, SF and CA, two well-studied, non-toxic NRF2 activators (27; 36), were tested for their ability to upregulate the NRF2 pathway in STZ-induced diabetic wild type mouse skin tissues. Diabetic mice were intraperitoneally injected with corn oil (vehicle control), 12.5 mg/kg SF, or 50 mg/kg CA every other day one week before and two weeks after wound surgery (Fig.
Untreated diabetic mice (STZ, n=5) had lower body weights (Fig. 2B) and higher blood glucose levels (Fig. 2C) than non-diabetic mice (Con, n=8) at 4 and 6 weeks after STZ injection, as expected. Treatment of diabetic mice with SF (STZ+SF, n=5) or CA (STZ+CA, n=6) reversed body weight loss during the initial phase of diabetes (Fig. 2B), but failed to decrease blood glucose levels (Fig. 2C). Immunoblotting demonstrated that SF and CA strongly upregulated the protein expression of NRF2 and its target genes HO-1, AKR1C1, and NQO1 in diabetic mouse skin tissue lysates (Fig. 2D). IHC also confirmed the high level of NRF2 and HO-1, which are mainly expressed in the epidermal layer of perilesional skin tissues in diabetic mice treated with SF or CA (Fig. 2E). These results indicate that SF and CA are able to activate the NRF2 pathway in mouse skin.

**Pharmacological NRF2 activation accelerates wound closure in STZ mice.** The effect of pharmacological NRF2 activation by SF and CA in diabetic wound healing was next investigated. The wounds of STZ mice healed slower than those of Con mice, but treatment of STZ mice with either SF or CA accelerated wound closure (Fig. 3A and B). Interestingly, the wound closure of STZ+SF mice was even faster than that of the Con mice during the first 7 days (Fig. 3B). Histological examination showed that the diameter of wounds at day 14 post-surgery in untreated STZ mice was the widest of all, while SF and CA markedly reduced the diameters of wounds to resemble that of the Con mice (Fig. 3C). However, non-diabetic mice treated with either SF or CA do not show accelerated wound closure (data not shown). These results indicate that NRF2 activation by SF or CA promotes wound healing in STZ mice.
SF and CA modulate the expression of TGF-β1 and MMP9, alleviate oxidative DNA damage, and decrease apoptosis of skin tissues in STZ mice. To investigate the mechanisms by which SF and CA improve wound healing in diabetic mice, their effects on proliferation and apoptosis were studied. The expression of TGF-β1 and MMP9, which are expressed by keratinocytes and play crucial roles during the proliferative and remodeling wound healing phases, was detected by immunoblotting and IHC. Wound skin tissues of STZ mice had lower TGF-β1 protein levels in the epidermal layer than Con mice, and STZ+SF or STZ+CA mice had TGF-β1 expression restored to levels comparable to those of Con mice (Fig. 4A and B). In contrast, STZ mice had the highest expression of MMP9 in all skin layers, but treatment with SF or CA restored its expression levels to those of Con mice. Therefore, the low TGF-β1 and high MMP9 protein expression in STZ mice might partly explain their delayed wound healing. IHC showed that wound skin tissues from STZ mice had the highest expression of 8-oxo-dG, but treatment with SF or CA greatly alleviated the oxidative DNA damage (Fig. 4B). TUNEL analysis showed that while wound skin tissues of STZ mice had a large extent of apoptosis, SF and CA treatments reduced apoptosis (Fig. 4B).

Next, digital infrared thermal imaging was used to measure temperature gradients between wound areas and the surrounding healthy tissue. This noninvasive and high-resolution technique is used to assess hemodynamic and neurogenic variations in the tissues of patients with skin diseases or diabetes mellitus; previous studies have identified that high temperature gradients may predict bad prognosis for diabetic patients with foot ulcers (37-39). Here, thermal imaging showed that the wound temperature gradients of STZ mice were greater than Con mice; however, SF and CA lowered the gradients (Fig. 4C). Collectively, these results indicate that SF
and CA promote diabetic wound healing by modulating the expression of TGF-β1 and MMP9, alleviating oxidative stress damage, and decreasing apoptosis of wound skin tissues in STZ mice.

**SF and CA activate the NRF2 pathway, modulate the expression of MMP9 and TGF-β, and alleviate oxidative stress in human keratinocytes under hyperglycemic condition.** To further understand the molecular mechanisms of NRF2-dependent acceleration of diabetic wound healing, *in vitro* studies were performed using an immortalized human keratinocyte cell line (HaCaT). HaCaT cells were first cultured in low glucose (LG) media for 2 weeks and then switched to high glucose (HG) media to mimic diabetic hyperglycemic conditions. Similar to the results reported above, culturing HaCaT cells under HG medium for 48 h activated the NRF2 pathway, as shown by an increase in NRF2 protein levels and further NRF2 induction by 5 µmol/L SF or 20 µmol/L CA treatment (Fig. 5A). Similarly, HO-1, AKR1C1, and NQO1 protein levels also increased in HG and were further induced by treatment with SF and CA (Fig. 5A). However, in HG+NRF2-siRNA cells, NRF2 pathway protein levels decreased to a level comparable to that of LG+Con-siRNA cells (Fig. 5B).

Since the expression of MMP9 and TGF-β1 was altered in the epidermal layer of diabetic mouse skin tissues, their expression in HaCaT cells was also investigated. Cells in HG medium had high intracellular MMP9 as well as higher activity of the secreted MMP9 (Fig. 5C and E). Moreover, NRF2 negatively modulated the expression and activity of MMP9 in HG, as it decreased with SF or CA treatment but increased in HG+NRF2-siRNA (Fig. 5D and F). Conversely, HG did not significantly increase the secretion of TGF-β1 with respect to LG, but there seems to be a positive correlation between extracellular TGF-β1 secretion and NRF2 levels in cells in HG medium (Fig. 5G and H).
Oxidative stress was also higher in cells in HG medium than in LG, and as predicted, activation of NRF2 by SF or CA reduced ROS levels significantly (Fig. 5I), whereas knockdown of NRF2 further increased ROS levels in HG cells (Fig. 5J). All these results indicate that HG induces oxidative stress, activates the NRF2 pathway, and alters the expression of MMP9 in keratinocytes. Furthermore, these results demonstrate the beneficial effects of pharmacological NRF2 activation in alleviating oxidative stress, inducing TGF-β1, and decreasing MMP9 of keratinocytes in HG conditions to promote diabetic wound healing.

**NRF2 activation promotes keratinocyte migration and proliferation, and decreases apoptosis.** To further confirm that wound healing was accelerated by NRF2 activation, an *in vitro* wound healing assay was performed to mimic reepithelialization through keratinocyte migration. HaCaT cell migration was significantly inhibited in cells in HG medium, which resulted in prolonged wound closure time, while SF and CA treatments significantly accelerated wound closure in HG medium (Fig. 6A), whereas HG+NRF2-siRNA cells had the slowest migration and the most delayed wound closure (Fig. 6B). These results demonstrate that pharmacological NRF2 activation can reverse impaired keratinocyte migration induced by HG.

Since keratinocyte proliferation also affects reepithelialization, the growth rate of HaCaT cells was assessed. Cell proliferation was slower in cells in HG, but was modestly induced by activation of NRF2 in HG+SF or HG+CA, and greatly decreased in HG+NRF2-siRNA cells (Fig. 7A and B). To further confirm this result, the cell proliferation marker Ki67 was detected by immunofluorescence. Accordingly, Ki67 expression in cells in HG was lower than in LG, and treatment with SF or CA could restore Ki67 expression (Fig. 7C), while in HG+NRF2-siRNA Ki67 expression further decreased (Fig. 7D), demonstrating that NRF2 activation positively
modulates keratinocyte proliferation. In contrast, increased levels of apoptosis were detected in cells in HG, and pharmacological NRF2 activation negatively modulated apoptosis (Fig. 7E and F). Collectively, these results demonstrate that hyperglycemia reduces keratinocyte proliferation but increases apoptosis, and these effects can be attenuated by activation of the NRF2 pathway.

**Discussion**

In the current study the essential role of NRF2 in diabetic wound healing was demonstrated. First, perilesional skin tissues from diabetic and normoglycemic patients were analyzed, finding that diabetic tissues were under more severe oxidative stress than normal wound skin tissues, as demonstrated by greater oxidative DNA damage, apoptosis, and compensatory NRF2 pathway activation. These same pathological alterations were observed in wounds in a STZ-induced diabetes mouse model, suggesting that Nrf2 contributes to diabetic wound healing. Therefore, the possibility of pharmacological NRF2 activation in a pre-treatment (before wounding) scheme was explored. The therapeutic potential of pharmacological NRF2 activation to restore normal wound healing was demonstrated in STZ-induced diabetic mice. To offer some molecular mechanistic insight, *in vitro* experiments with keratinocytes were performed, and the results further confirmed that pharmacological NRF2 activation contributes to important events of wound healing including oxidative stress attenuation, promotion of proliferation and migration, and decreased apoptosis under high glucose.

Oxidative stress is prevalent in diabetes. High oxidative stress causes damage to proteins, lipids, and DNA in the cells, which may ultimately lead to cell death and consequent tissue dysfunction. In our model, diabetes (in both human and mouse skin tissue samples) causes oxidative stress, as measured by oxidative DNA damage (8-oxo-dG) and increased apoptosis.
(TUNEL). This diabetes-induced higher basal oxidative stress, in addition to oxidative stress induced by the wounding and inflammation results in impaired (slower) diabetic wound healing. In response to oxidative stress, cells activate Nrf2 to contend and repair the damage; however, the damage has already occurred. On the other hand, pharmacological activation of Nrf2 before wounding ensures that 1) diabetes-induced oxidative stress levels are reduced, and 2) wounding-associated oxidative stress levels don’t peak because the cells are already primed to contend this damage. Therefore, pharmacological Nrf2 activation ensures the cells are protected (less damage, less apoptosis) and also regulates the expression of other proteins important for wound healing (MMP9, TGFβ, migration and proliferation-related genes) through direct or indirect mechanisms, some of which and the effects of different oxidative stress levels on Nrf2 signaling/wound healing remain to be elucidated in future studies.

Although this is the first study reporting the crucial role of NRF2 in diabetic wound healing, its role in non-diabetic wound healing has been previously explored. A study showed that NRF2 promotes epithelial cell proliferation and migration (23); however, another study found no apparent abnormalities in the wound healing process of Nrf2−/− mice other than prolonged inflammation in the later stages of wound repair compared to Nrf2+/+ mice (40). Using a transgenic mouse expressing a dominant negative NRF2 mutant in the epidermis the same group showed that NRF2 is not essential for normal wound healing (41). Consistent with their findings, we also observed that NRF2 activation by SF or CA does not promote wound healing in non-diabetic mice, indicating NRF2 activation has no benefit in normal wound healing. In contrast, we observed delayed wound healing, increased oxidative stress, and apoptosis in Nrf2−/− diabetic mice compared to wild type (Supplementary Fig. 1), demonstrating that NRF2 signaling is essential in diabetic wound healing. This may be due to the fact that diabetic wounds have
higher oxidative stress than normal wounds, resulting in increased DNA oxidative damage and apoptosis. Indeed, we observed the beneficial effect of pre-treatment of mouse diabetic skin with NRF2 activators (SF and CA) in reducing oxidative stress and improving diabetic wound healing.

Keratinocytes are the predominant cell type in the epidermal layer and play an essential role during the wound healing process (42; 43). Our study showed that HaCaT cells in HG medium (used to mimic the hyperglycemic condition of diabetic patients) had reduced migration and proliferation, consistent with other studies (44; 45). Furthermore, we provide evidence suggesting that pharmacological activation of NRF2 promotes keratinocyte proliferation and migration but inhibits apoptosis. For keratinocyte migration to occur hemidesmosomes must disassemble and the extracellular matrix has to be remodeled. A recent study found that NRF2 indirectly downregulates the desmosomal protein DSC2 (46), which might explain why migration is enhanced after NRF2 induction. In contrast, other studies found that NRF2 inhibits migration in cancer cell lines (20; 47). Undoubtedly, future investigations will help clarify the cell-type specific effects of NRF2 expression on cell migration.

Delayed diabetic wound healing is characterized by an increase in matrix metalloproteinases and a reduction in some growth factors, in particular TGF-β1, in skin tissue (48-50). Higher MMP9 activity or expression in wound fluid and lower TGF-β1 expression were identified in biopsy skin samples of human diabetic foot ulcers, which associated with poor wound healing (51; 52). Consistently, we identified lower TGF-β1 expression and higher MMP9 expression and activity in STZ mice. The expression and activity of MMP9 were also higher in cells grown in HG medium than in LG. However, there was no difference in the levels of TGF-β1 between the two conditions, which could be due to the fact that in vivo wound healing is a very complex process and many pathological processes associated to diabetes (hyperglycaemia,
ischemia, hypoxia, advanced glycation end products, etc.) may act as contributing factors. Our results suggest that pharmacological activation of NRF2 signaling positively modulates TGF-β1 and negatively modulates MMP9 in keratinocytes during diabetic wound healing.

Undoubtedly, many additional factors contribute to the impaired healing of diabetic foot ulcers. Neuropathy and higher skin temperatures resulting from abnormal microvasculature blood flow predispose the diabetic foot to ulceration (53; 54). In this study, using IR we found that STZ mice had higher wound temperatures that correlated to their slower closure and upon treatment with SF or CA the temperatures decreased, further supporting that this technique could be used to predict therapeutic effectiveness in wound healing of diabetic patients.

In summary, our findings define for the first time a novel function for NRF2 in the diabetic wound healing process. Furthermore, this study sets the basis for clinical assessment and application of NRF2 activators in treating diabetic skin ulcers. However, the benefit of pharmacological NRF2 activation observed in this study through systemic administration of NRF2 inducers is multi-factorial, and the exact mechanisms by which NRF2 may modulate inflammation, granulation tissue, vascular, and neural functions in diabetic conditions remain to be investigated in the future. In addition, a topical NRF2 activator-based formulation applied to diabetic patients’ skin before ulceration could provide a practical therapeutic intervention.
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Author contributions. M.L., M.B., P.W., G.T.W., H.Z., and D.D.Z. designed the study. R.Z., S.Z., and H.Z. acquired the human tissue samples. M.L., T.J., and Q.W. performed the experiments. M.L. and M.R.V. wrote the manuscript. G.T.W., H.Z., and D.D.Z. supervised the overall study. D.D.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Figure 1. Perilesional skin tissues of diabetic patients are under severe oxidative damage that activates the NRF2-mediated antioxidant response. Human skin tissue samples from 11 normoglycemic and 12 diabetic patients (see Supplementary Table 1 for details) were fixed and paraffin-embedded; the tissue sections were subjected to H&E staining (A,B) and IHC analysis (C-J) with the indicated antibodies (magnification: 200x). Apoptotic cells in the tissue were detected by transferase-mediated dUTP nick-end labeling (TUNEL) assay (K,L) (magnification: 100x). Representative images from the perilesional skin tissues of wounds are shown. Scale bar, 100 µm.

Figure 2. SF and CA activate NRF2 in skin tissues of STZ mice. (A) Time line for treatments, surgery, and wound healing assessment in mice. Diabetic mice were generated by STZ injections as described above; non-diabetic controls were injected with sodium citrate instead of STZ. Three weeks later, all mice became diabetic (FGL ≥250 mg/dL) and were randomly allocated into STZ or STZ+treatment (SF or CA) groups. All animals received compounds or corn oil every other day until the end of the experiment. Wound surgeries were done after one week of compound treatments; 2 weeks later, the wound skin tissues were harvested. Groups: non-
diabetic control (Con, n=8); diabetic: untreated (STZ, n=5), SF-treated (STZ+SF, n=5), CA-treated (STZ+CA, n=6). (B) Relative body weight and (C) blood glucose concentration. Data were analyzed by ANOVA and Tukey post hoc test. Results are expressed as means ± SEM (n=5-8), *P<0.05 compared to Con, #P<0.05 compared to STZ. (D) Immunoblots of NRF2, HO-1, AKR1C1, NQO1, and actin using mouse wound skin tissues (each lane contains wound skin tissue lysates from an individual mouse, n=3 per group). (E) IHC analysis of NRF2 and HO-1 using mouse wound skin tissues. Representative images from each group are shown (magnification: 100x).

**Figure 3. Pharmacological NRF2 activation accelerates wound closure in STZ mice.** Two wounds were done in the backs of mice (n=5-8) as described in Methods. (A) Representative photographs of wounds of mice in different groups at the indicated time points. (B) Wound closure. All mice had two wounds made in their backs and the wounds were photographed at the indicated time points before the skin tissues were harvested at day 14. The area of the two wounds was measured at the indicated time points to calculate wound closure (the percentage of wound that healed) at the indicated time points. Data were analyzed by ANOVA and Tukey post hoc test, results are expressed as means ± SEM (n=10-16). *P<0.05 compared to Con, #P<0.05 compared to STZ. (C) Pathological assessment and diameter of mouse wound skin tissues 14 days after wound surgery. A representative image from one mouse per group is shown, the borders of the wound are indicated by dotted lines (magnification: 40x).
Figure 4. SF and CA modulate the expression of TGF-β1 and MMP9, alleviate oxidative DNA damage, and decrease apoptosis of skin tissues in STZ mice. (A) Immunoblots of TGF-β1, MMP9, and actin using mouse wound skin tissue lysates. (B) IHC analysis of wound skin tissues using the indicated antibodies, as well as apoptosis by TUNEL assay. A representative image from each group is shown (magnification: 100x). (C) Infrared imaging of wound skin tissues. Representative photographs of wounds at the indicated days post-surgery (left) and quantification of temperature changes (ΔT) between wound area and surrounding healthy regions in the different treatment groups (right) are shown. Data were analyzed by ANOVA and Tukey post hoc test, results are expressed as means ± SEM (n=10-16). *P<0.05 compared to Con, #P<0.05 compared to STZ.

Figure 5. SF and CA activate the NRF2 pathway, modulate the expression of MMP9 and TGF-β1, and alleviate oxidative stress in human keratinocytes under hyperglycemic condition. (A,B) Immunoblots of NRF2, HO-1, AKR1C1, NQO1 and Actin. HaCaT cells were incubated in either low glucose (LG) or high glucose (HG) medium for two days. HG cells were treated with 5 μmol/L SF or 20 μmol/L CA (HG+SF, HG+CA) for 48 h (A) or were transfected with the indicated siRNA (HG+ConsiRNA, HG+NRF2siRNA) for 72 h (B). Cell lysates were subjected to immunoblot analysis. (C-F) Immunoblots of MMP9 and zymography of secreted MMP9. HaCaT cells were incubated and treated as above, during the last 24 h HaCaT cells were switched from medium with 10% FBS to no FBS. (C,D) Cells were harvested and subjected to immunoblot analysis. (E,F) In another experiment, the medium was harvested to detect proteolytic activity of equal concentration of MMP9 by gelatin zymography; the cells were harvested to show equal loading by immunoblots. (G,H) Immunoassay of TGF-β1 secreted to the medium. HaCaT cells were treated as above and the medium was harvested to detect the
extracellular TGF-β1 levels. The mean values were used and normalized to the LG (G) or LG+ConsiRNA (H) group, represented as bar graphs. Data were analyzed by ANOVA and Tukey post hoc test, results are expressed as means ± SEM (n=4). \#P<0.05 compared to HG or HG+ConsiRNA. (I,J) ROS detection and quantification. Similarly treated HaCaT cells were subjected to DCF/flow cytometry analysis for ROS detection. The mean fluorescence values were used and normalized to the LG (I) or LG+ConsiRNA (J) group, represented as bar graphs. Data were analyzed by ANOVA and Tukey post hoc test, results are expressed as means ± SEM (n=3), *P<0.05 compared to LG or LG+ConsiRNA, \#P<0.05 compared to HG or HG+ConsiRNA.

Figure 6. NRF2 activation promotes keratinocyte migration. (A,B) In vitro wound healing assay of keratinocytes. HaCaT cells were incubated in either low glucose (LG) or high glucose (HG) medium for two days. Cells in HG were treated with 5 µmol/L SF or 20 µmol/L CA (HG+SF, HG+CA) for 24 h before removal of PDMS slab to generate gaps (A) or were transfected with the indicated siRNA (HG+ConsiRNA, HG+NRF2siRNA) for 48 h, followed by removal of PDMS slab (B). Cells were incubated with fresh medium without or with SF or CA everyday up to 72 h. Representative cell images from each group in the indicated time points after removal of PDMS slab are shown; the white dotted lines represent the wound boundary (left panel). Quantification of wound healing is shown (right panel). Data were analyzed by ANOVA and Tukey post hoc test, results are expressed as means ± SEM (n=4). *P<0.05 compared to LG or LG+ConsiRNA group, \#P<0.05 compared to HG or HG+ConsiRNA. (C,D) Cell growth index.
Figure 7. NRF2 activation promotes keratinocyte proliferation and decreases apoptosis. 

(A, B) Proliferation was assessed as cell growth index. Similarly treated (A) or siRNA-transfected (B) HaCaT cells were monitored for cell growth up to 72 h in real time. Data are expressed as means ± SEM (n=3). (C, D) Ki67 immunofluorescence images (top) and quantification of fluorescence intensity (bottom graph). Similarly treated (C) or siRNA-transfected (D) HaCaT cells were subjected to immunofluorescence analysis with Ki67 antibodies. The relative Ki67 expression was quantified, analyzed by ANOVA and Tukey post hoc test, results are expressed as means ± SEM (n=3). *P<0.05 compared to LG or LG+ConsiRNA, #P<0.05 compared to HG or HG+ConsiRNA. (E, F) In situ cell death assessment by TUNEL assay (top) and quantification (bottom graph). Similarly treated (E) or siRNA-transfected (F) HaCaT cells were subjected for TUNEL analysis. For the positive control, cells were treated with 20 µmol/L cisplatin for 24 h. Relative cell apoptosis was quantified, analyzed by ANOVA and Tukey post hoc and the results are expressed as means ± SEM (n=3). *P<0.05 compared to LG or LG+ConsiRNA, #P<0.05 compared to HG or HG+ConsiRNA.
Figure 1. Perilesional skin tissues of diabetic patients are under severe oxidative damage that activates the NRF2-mediated antioxidant response.

279x361mm (300 x 300 DPI)
Figure 2. SF and CA activate NRF2 in skin tissues of STZ mice.
279x361mm (300 x 300 DPI)
Figure 3. Pharmacological NRF2 activation accelerates wound closure in STZ mice.

279x361mm (300 x 300 DPI)
Figure 4. SF and CA modulate the expression of TGF-β1 and MMP9, alleviate oxidative DNA damage, and decrease apoptosis of skin tissues in STZ mice.
Figure 5. SF and CA activate the NRF2 pathway, modulate the expression of MMP9 and TGF-β1, and alleviate oxidative stress in human keratinocytes under hyperglycemic condition.
Figure 6. NRF2 activation promotes keratinocyte migration.

297x420mm (300 x 300 DPI)
Figure 7. NRF2 activation promotes keratinocyte proliferation and decreases apoptosis.
SUPPLEMENTAL FIGURE LEGEND

Supplementary Figure 1. Deletion of Nrf2 delays wound healing in an STZ-induced diabetic mouse model. (A) Time line for treatments, surgery and wound healing assessment. Nrf2<sup>+</sup>/ and Nrf2<sup>-/-</sup> mice at week 0 (8 weeks of age) received STZ intraperitoneal injections in 5 consecutive days to induce diabetes. 4 weeks later, all mice had FGL ≥250 mg/dL and were considered diabetic. Each diabetic Nrf2<sup>+/+</sup> (n=5) or Nrf2<sup>-/-</sup> (n=6) mouse had two wounds made in its back. Wounds were photographed at the indicated time points before the skin tissues were harvested 2 weeks later (at day 14 post-surgery). (B) Representative photographs of wounds of diabetic Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice. The day after the wound surgery is indicated at the bottom. (C) Wound closure. Wound area was quantified and presented as the percentage of wound that healed up to 14 days after wound surgery. Data are expressed as means ± SEM (n=10-12), comparisons of diabetic Nrf2<sup>+/+</sup> vs. Nrf2<sup>-/-</sup> mice were done with a Student’s t test. *P<0.05 compared to diabetic Nrf2<sup>-/-</sup> mice. (D) IHC analysis of wound skin tissues harvested at day 14 with the indicated antibodies; apoptotic cells in the tissue were detected by TUNEL assay (magnification: 100x).
Supplementary figure 1

A

0 1 2 3 4 5 6
Time (week)

i.p. STZ

Wound surgery (F0L2 250 mg/dL)

Harvest skin

Wound healing

B

Nrf2−/−

Nrf2+/−

Time (day)

0 5 10 15

C

Wound closure (%)

Nrf2−/−

Nrf2+/−

Time (day)

0 2 4 6 8 10 12 14

D

NRF2

HO-1

8-oxo-dG

Nrf2−/−

Nrf2+/−

TGF-β1

MMP9

TUNEL

Nrf2−/−

Nrf2+/−
Supplementary Table 1: Diabetic patients’ characteristics and baseline measurements

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Diabetes (n=12)</th>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td>Female</td>
<td>4 (33.3%)</td>
</tr>
<tr>
<td>Male</td>
<td>8 (66.7%)</td>
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<tr>
<td>Specimen from foot</td>
<td>12 (100.0%)</td>
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<tr>
<td>Type 1 diabetes</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>11 (91.7%)</td>
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<tr>
<td>Diabetes duration, years</td>
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<tr>
<td>Diabetic ulcers duration, days</td>
<td>92.9 ± 29.7</td>
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<tr>
<td>Age, years</td>
<td>66.3 ± 3.3</td>
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<tr>
<td>Wound area, cm²</td>
<td>14.3 ± 3.7</td>
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<tr>
<td>HbA1c, % (mmol/mol)</td>
<td>9.8 ± 1.6 (84 ± 17.5)</td>
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<tr>
<td>FPG, mg/dL</td>
<td>8.1 ± 0.6</td>
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<tr>
<td>2-h PPG, mg/dL</td>
<td>12.3 ± 0.8</td>
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<tr>
<td><strong>Medications</strong></td>
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<tr>
<td>Insulin</td>
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<tr>
<td>Acarbose</td>
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</tr>
<tr>
<td>ACEI or ARB</td>
<td>6 (50.0%)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>3 (25.0%)</td>
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</tbody>
</table>

Continuous data are presented as Mean ± SEM and categoric data as n (%). HbA1c, glycated hemoglobin A1c. FPG, fasting plasma glucose. PPG, postprandial plasma glucose. ACEI, angiotensin-converting enzyme inhibitor. ARB, angiotensin receptor blockers.