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An Essential Role of NRF2 in Diabetic Wound Healing

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The high mortality and disability of diabetic nonhealing 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 patients with diabetes are under more severe oxidative stress and display higher activation of the nuclear factor-E2-related factor 2 (NRF2)-mediated antioxidant response than perilesional skin tissues from normoglycemic patients. In a streptozotocin-induced diabetes mouse model, *Nrf2*^{-/-} mice have delayed wound closure rates compared with *Nrf2*^{+/+} mice, which is, at least partially, due to greater oxidative DNA damage, low transforming growth factor-β1 (TGF-β1) and high matrix metalloproteinase 9 (MMP9) expression, and increased apoptosis. More importantly, pharmacological activation of the NRF2 pathway significantly improves diabetic wound healing. In vitro experiments in human immortalized keratinocyte 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 nonhealing skin ulcers are a major cause of disability and mortality in the population with diabetes (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 the 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 that regulate angiogenesis, granulation tissue formation, ECM remodeling, reepithelialization, 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 nuclear factor-E2-related factor 2 (NRF2) regulates the adaptive response to exogenous and

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endogenous oxidative stress (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 current study aimed to explore the role of NRF2 in diabetic wound healing. Perilesional skin tissue samples from patients with diabetes and ulcers 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 patients with diabetes.

RESEARCH DESIGN AND METHODS

Chemicals, Antibodies, and Cell Culture

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

Human immortalized keratinocytes (HaCaT) were obtained from Dr. Tim Bowden (Arizona Cancer Center). Cells were grown in DMEM (Cellgro, Manassas, VA) containing low glucose (LG) (5.5 mmol/L) and 10% FBS at 37°C with 5% CO₂. For the experiments, cells were starved in serum-free LG for 24 h and 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 μ mol/L SF or 20 μ mol/L CA.

Small Interfering RNA 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. In brief, cells were maintained in LG or HG for 2 days and then transfected with siRNA for migration, proliferation, and apoptosis experiments. For reactive oxygen species (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 (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 or 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 Xinqiao Hospital, Third Military Medical University, Chongqing, People's Republic of 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. In brief, 3 weeks after STZ injections, fasting glucose levels (FGLs) (4 h fast) were measured and all mice had FGL \geq 250 mg/dL, and thus were considered diabetic and included in the study (see Fig. 2A for a detailed timeline). Nondiabetic 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 2 days until skin tissues were harvested (27,29). One week after the treatments, the mice were anesthetized and 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, and the other half was used for protein extraction. Mice that were too weak or developed wound complications were excluded from the analysis.

Immunohistochemistry, Immunoblotting, TGF- β 1 Immunoassay, and Gelatin Zymography

Human and mouse skin tissue morphology was assessed by hematoxylin-eosin (H-E) staining (Vector, Burlingame, CA). Immunohistochemistry (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- β 1 and MMP9, cells were incubated for 24 h in serum-free DMEM before harvesting the medium. TGF- β 1 was measured using the Quantikine Human TGF- β 1 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- β 1 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). In brief, UV-sterilized polydimethylsiloxane (PDMS) blocks (1 mm \times 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 μ mol/L SF or 20 μ mol/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.

ROS Measurement, Cell Proliferation, and Apoptosis

ROS were measured in HaCaT cells using DCF. In brief, the cells were seeded in LG or HG and treated with 5 μ mol/L SF or 20 μ mol/L CA every 24 h (33,34). After 48 h, the cells were switched into fresh medium containing 10 μ g/mL DCF and incubated for 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, 8,000 HaCaT cells/well were seeded in LG or HG, with or without NRF2 activators, and cell growth was monitored. 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 \pm SEM. Statistical tests were performed using GraphPad Prism 6.0 (La Jolla, CA). In vitro experiments were performed in triplicate. One-way ANOVA with Tukey post hoc test was applied to compare the means of three or more groups. Unpaired, two-sided Student *t* tests were used to compare the means of two groups. *P* < 0.05 was considered to be significant.

RESULTS

Perilesional Skin Tissues of Patients With Diabetes Are Under Severe Oxidative Stress That Activates the NRF2-Mediated Antioxidant Response

Perilesional skin tissues were collected from normoglycemic patients and patients with diabetes for pathological (H-E) and IHC analyses. Diabetic skin had more inflammatory cell infiltration and edema and less granulation tissue formation than normoglycemic skin, indicating an impaired wound healing process during 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 wound skin (Fig. 1C-H). These results suggest that skin tissues of patients with diabetes undergo severe oxidative damage that causes apoptosis and compensatory NRF2 pathway activation.

SF and CA Activate NRF2 in Skin Tissues of STZ Mice

On the basis of the observation that the wounds of patients with diabetes 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 with diabetic *Nrf2*^{+/+} mouse skin (Supplementary Fig. 1D). Moreover, diabetic *Nrf2*^{-/-} mouse skin had lower TGF- β 1 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, nontoxic 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

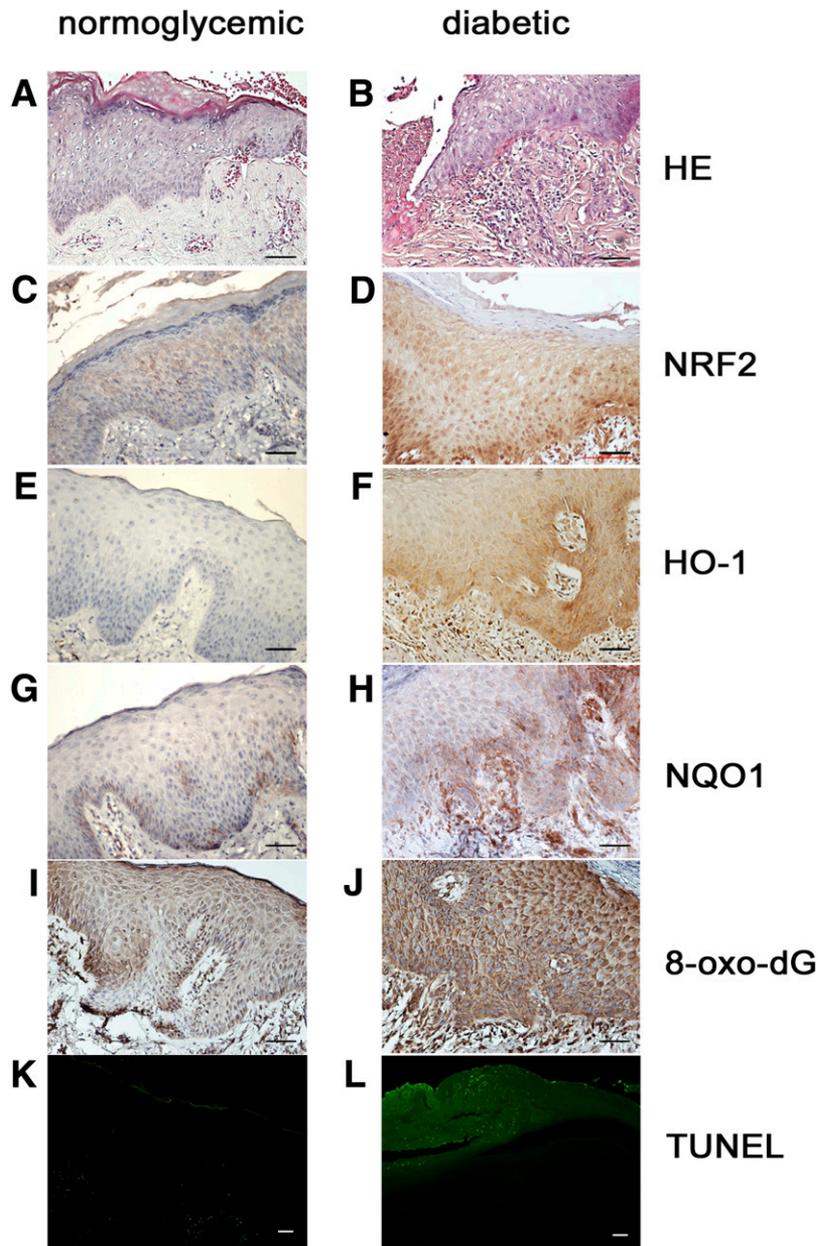


Figure 1—Perilesional skin tissues of patients with diabetes are under severe oxidative damage that activates the NRF2-mediated antioxidant response. Human skin tissue samples from 11 normoglycemic patients and 12 patients with diabetes (see Supplementary Table 1 for details) were fixed and paraffin embedded; the tissue sections were subjected to H-E staining (A and B) and IHC analysis (C–J) with the indicated antibodies (original magnification $\times 200$). Apoptotic cells in the tissue were detected by TUNEL assay (K and L) (original magnification $\times 100$). Representative images from the perilesional skin tissues of wounds are shown. Scale bar, 100 μm .

oil (vehicle control), 12.5 mg/kg SF, or 50 mg/kg CA every other day 1 week before and 2 weeks after wound surgery (Fig. 2A). Untreated diabetic mice (STZ, $n = 5$) had lower body weights (Fig. 2B) and higher blood glucose levels (Fig. 2C) than nondiabetic 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.

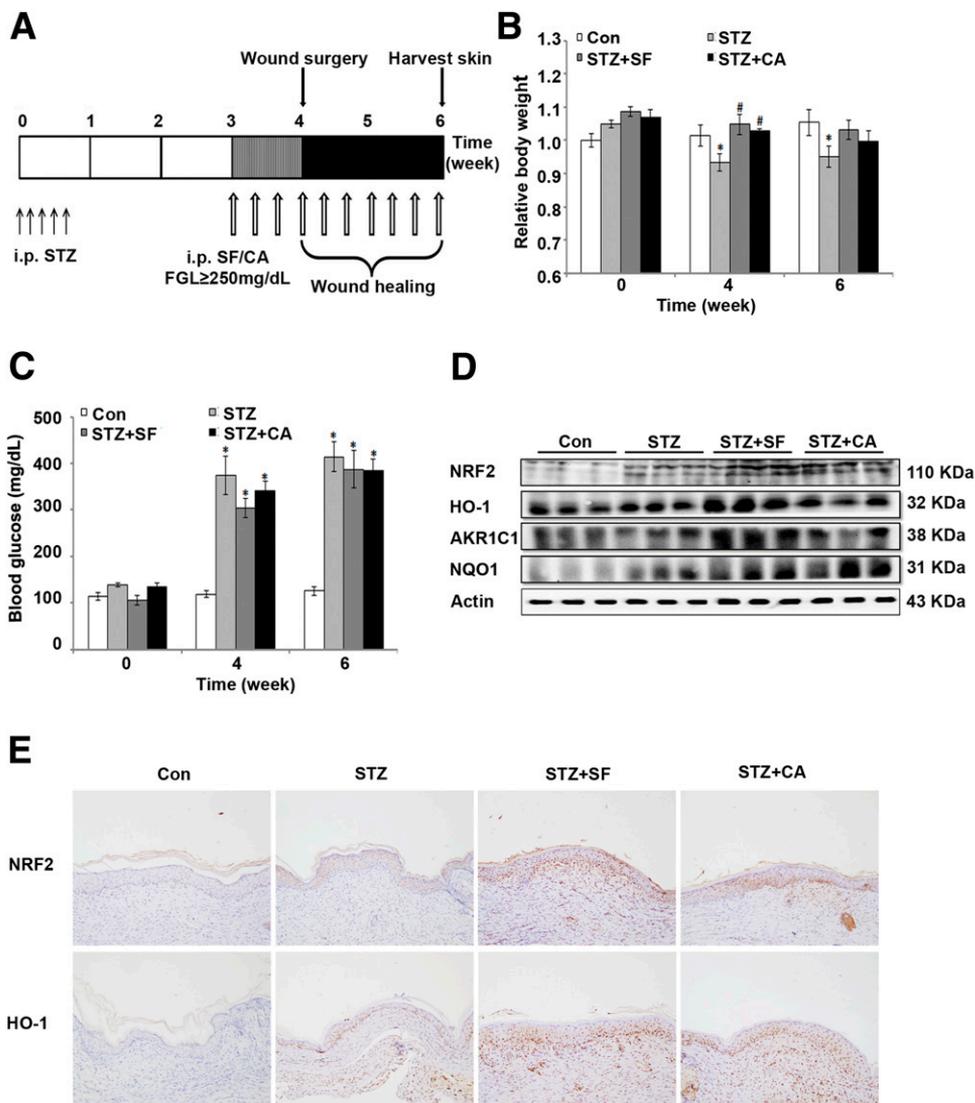


Figure 2—SF and CA activate NRF2 in skin tissues of STZ mice. **A**: Timeline for treatments, surgery, and wound healing assessment in mice. Diabetic mice were generated by STZ injections as described above; nondiabetic controls were injected with sodium citrate instead of STZ. Three weeks later, all mice became diabetic ($FGL \geq 250$ 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 performed after 1 week of compound treatments; 2 weeks later, the wound skin tissues were harvested. Groups: nondiabetic control (Con, $n = 8$); diabetic: untreated (STZ, $n = 5$), SF treated (STZ+SF, $n = 5$), and CA treated (STZ+CA, $n = 6$). Relative body weight (**B**) and blood glucose concentration (**C**). Data were analyzed by ANOVA and Tukey post hoc test. Results are expressed as means \pm SEM ($n = 5$ –8). * $P < 0.05$ compared with Con; # $P < 0.05$ compared with 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 (original magnification $\times 100$).

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 postsurgery in untreated STZ mice was the widest of all, whereas SF and CA markedly reduced the diameters of wounds to resemble that of the Con mice (Fig. 3C). However, nondiabetic 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.

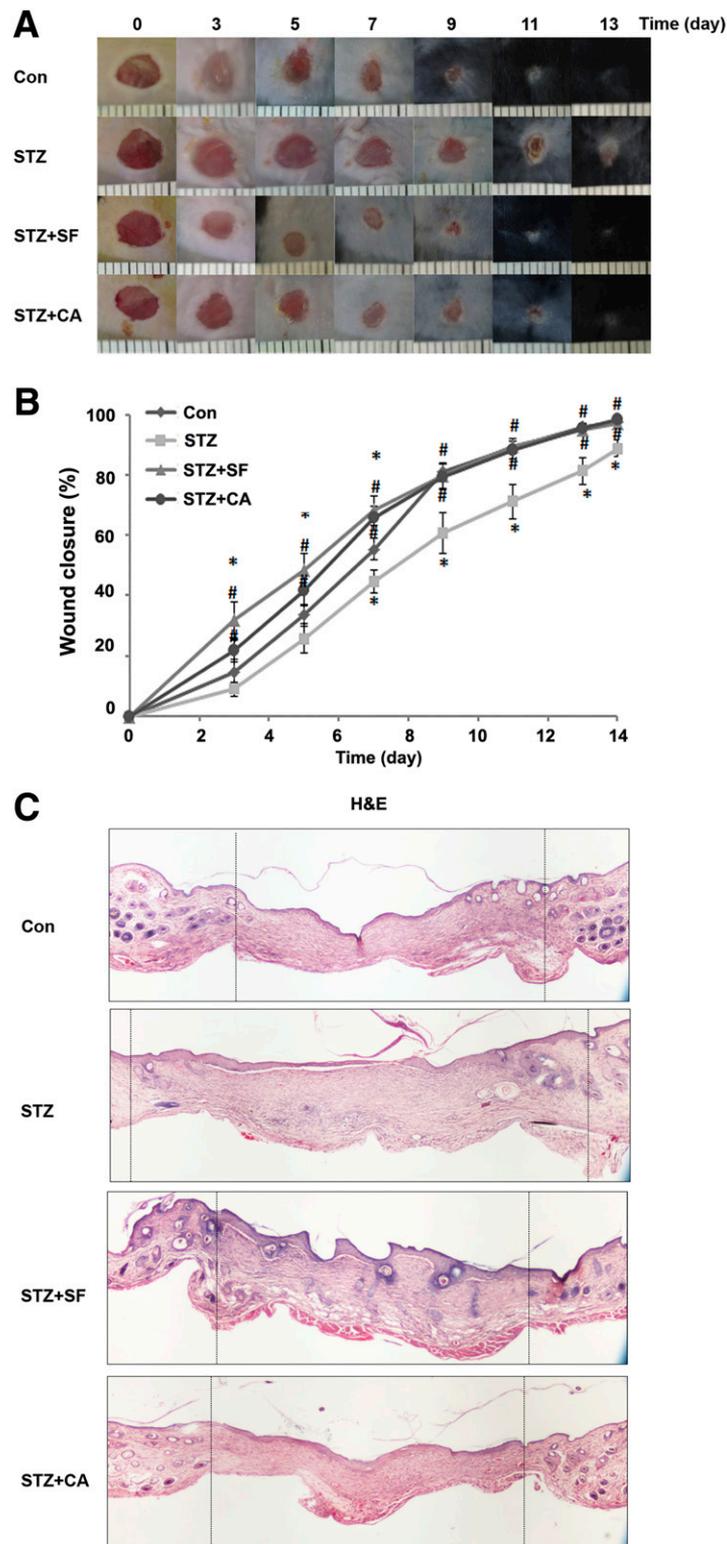


Figure 3—Pharmacological NRF2 activation accelerates wound closure in STZ mice. Two wounds were made in the backs of mice ($n = 5-8$) as described in RESEARCH DESIGN AND METHODS. **A**: Representative photographs of wounds of mice in different groups at the indicated time points. **B**: Wound closure. 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 \pm SEM ($n = 10-16$). * $P < 0.05$ compared with Con; # $P < 0.05$ compared with 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 (original magnification $\times 40$).

SF and CA Modulate the Expression of TGF- β 1 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- β 1 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- β 1 protein levels in the epidermal layer than Con mice, and STZ+SF or STZ+CA mice had TGF- β 1 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- β 1 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 whereas 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; previous studies have identified that high temperature gradients may predict a bad prognosis for patients with diabetes and 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- β 1 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 Conditions

To further understand the molecular mechanisms of NRF2-dependent acceleration of diabetic wound healing, *in vitro* studies were performed using an HaCaT cell line. HaCaT cells were first cultured in LG media for 2 weeks and then switched to 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 μ mol/L SF or 20 μ mol/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- β 1 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- β 1 with respect to LG, but there seems to be a positive correlation between extracellular TGF- β 1 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- β 1, 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), whereas in HG+NRF2-siRNA Ki67 expression further decreased (Fig. 7D), demonstrating that NRF2 activation positively modulates keratinocyte proliferation. In contrast, increased levels of apoptosis

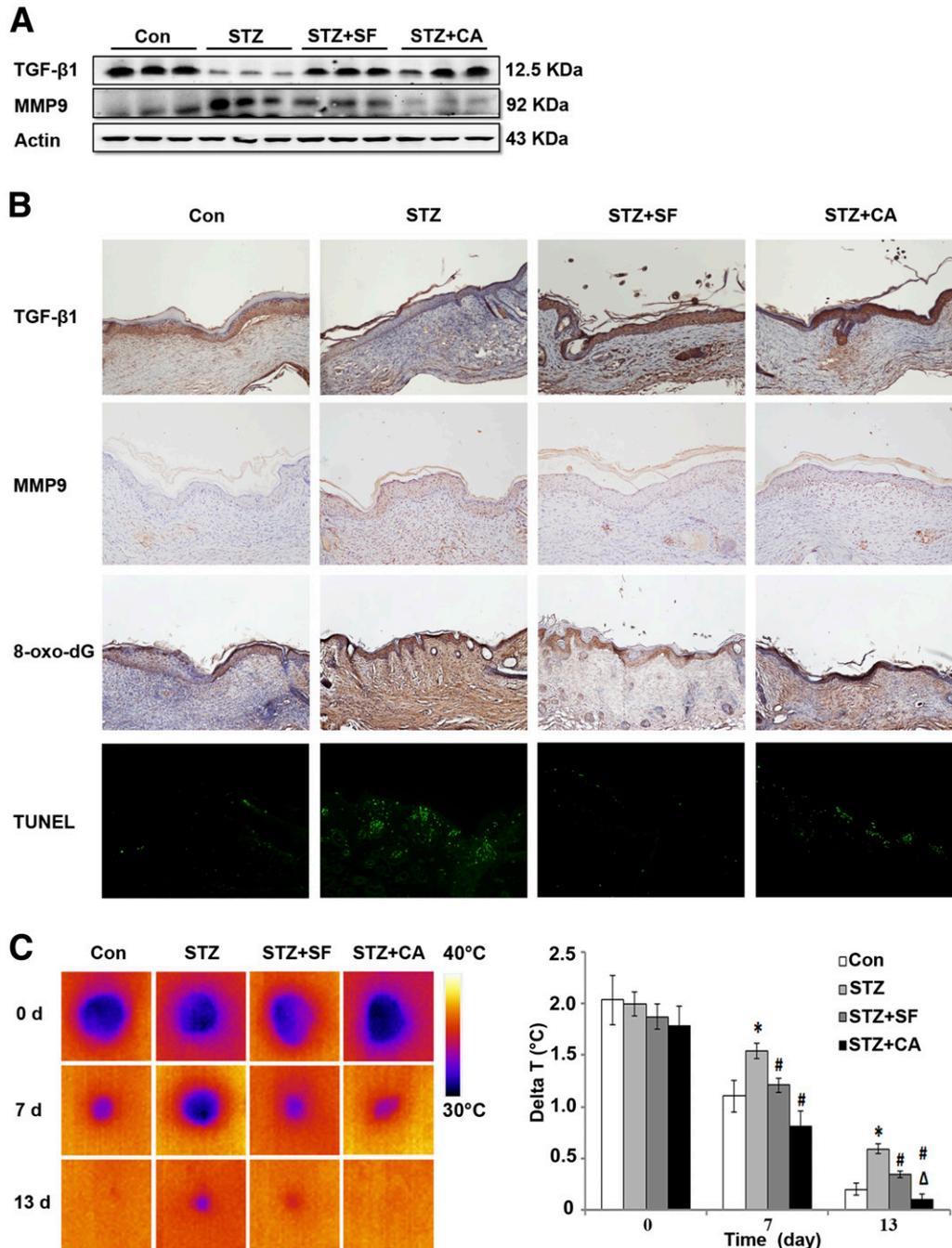


Figure 4—SF and CA modulate the expression of TGF-β1 and MMP9, alleviate oxidative DNA damage, and decrease apoptosis of skin tissues in STZ mice. **A:** Immunoblots of TGF-β1, MMP9, and actin using mouse wound skin tissue lysates. **B:** IHC analysis of wound skin tissues using the indicated antibodies, as well as apoptosis by TUNEL assay. A representative image from each group is shown (original magnification ×100). **C:** Infrared imaging of wound skin tissues. Representative photographs of wounds at the indicated days postsurgery (left) and quantification of temperature changes (Delta T) 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). d, day. *P < 0.05 compared with Con; #P < 0.05 compared with STZ; ΔP < 0.05 compared with STZ+SF.

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 normoglycemic patients and patients with diabetes were analyzed, finding that diabetic tissues were under more severe oxidative stress than normal wound

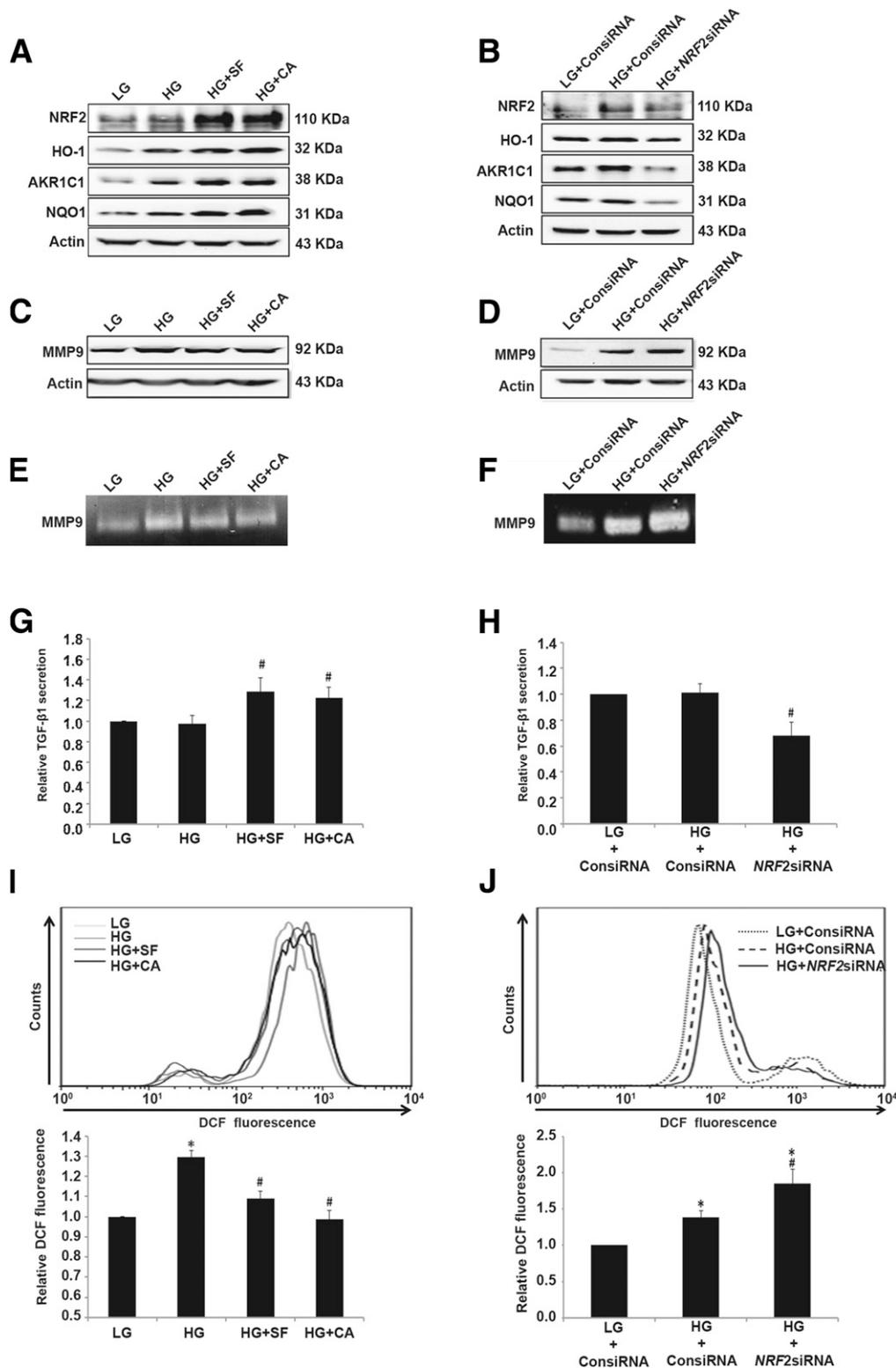


Figure 5—SF and CA activate the NRF2 pathway, modulate the expression of MMP9 and TGF-β1, and alleviate oxidative stress in human keratinocytes under hyperglycemic conditions. *A* and *B*: Immunoblots of NRF2, HO-1, AKR1C1, NQO1, and actin. HaCaT cells were incubated in either LG or HG medium for 2 days. HG cells were treated with 5 μmol/L SF or 20 μmol/L CA (HG+SF or HG+CA) for 48 h (*A*) or were transfected with the indicated siRNA (HG+Con-siRNA or HG+NRF2-siRNA) 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* and *D*: Cells were harvested and subjected to immunoblot analysis. *E* and *F*: In another experiment, the medium was harvested to detect proteolytic activity of equal concentration of MMP9 by gelatin zymography. *G* and *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

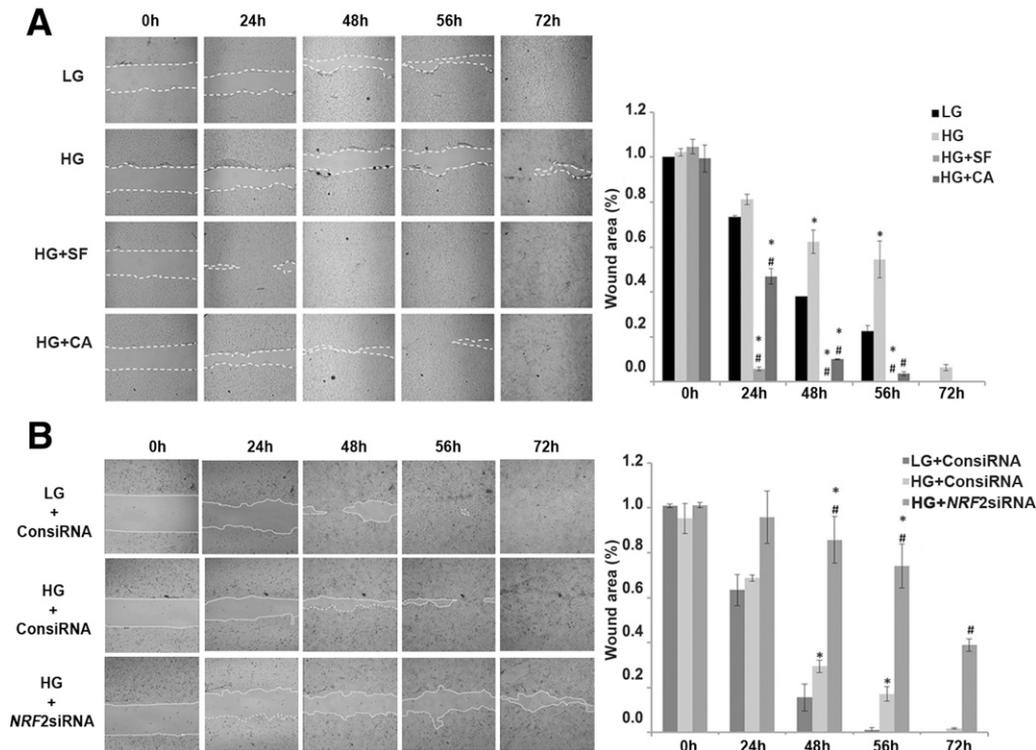


Figure 6—NRF2 activation promotes keratinocyte migration. *A* and *B*: In vitro wound healing assay of keratinocytes. HaCaT cells were incubated in either LG or HG medium for 2 days. Cells in HG were treated with 5 $\mu\text{mol/L}$ SF or 20 $\mu\text{mol/L}$ CA (HG+SF or HG+CA) for 24 h before removal of PDMS slab to generate gaps (*A*) or were transfected with the indicated siRNA (HG+Con-siRNA or HG+NRF2-siRNA) for 48 h, followed by removal of PDMS slab (*B*). Cells were incubated with fresh medium without or with SF or CA every day 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 panels). Quantification of wound healing is shown (right panels). Data were analyzed by ANOVA and Tukey post hoc test. Results are expressed as means \pm SEM ($n = 4$). * $P < 0.05$ compared with LG or LG+Con-siRNA group; # $P < 0.05$ compared with HG or HG+Con-siRNA.

skin tissues, as demonstrated by greater oxidative DNA damage, apoptosis, and compensatory NRF2 pathway activation. These same pathological alterations were observed in wounds in an STZ-induced diabetes mouse model, suggesting that NRF2 contributes to diabetic wound healing. Therefore, the possibility of pharmacological NRF2 activation in a pretreatment (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 HG.

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 do not peak because the cells are already

(G) or LG+Con-siRNA (*H*) group, represented as bar graphs. Data were analyzed by ANOVA and Tukey post hoc test. Results are expressed as means \pm SEM ($n = 4$). # $P < 0.05$ compared with HG or HG+Con-siRNA. *I* and *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+Con-siRNA (*J*) group, represented as bar graphs. Data were analyzed by ANOVA and Tukey post hoc test. Results are expressed as means \pm SEM ($n = 3$). * $P < 0.05$ compared with LG or LG+Con-siRNA; # $P < 0.05$ compared with HG or HG+Con-siRNA.

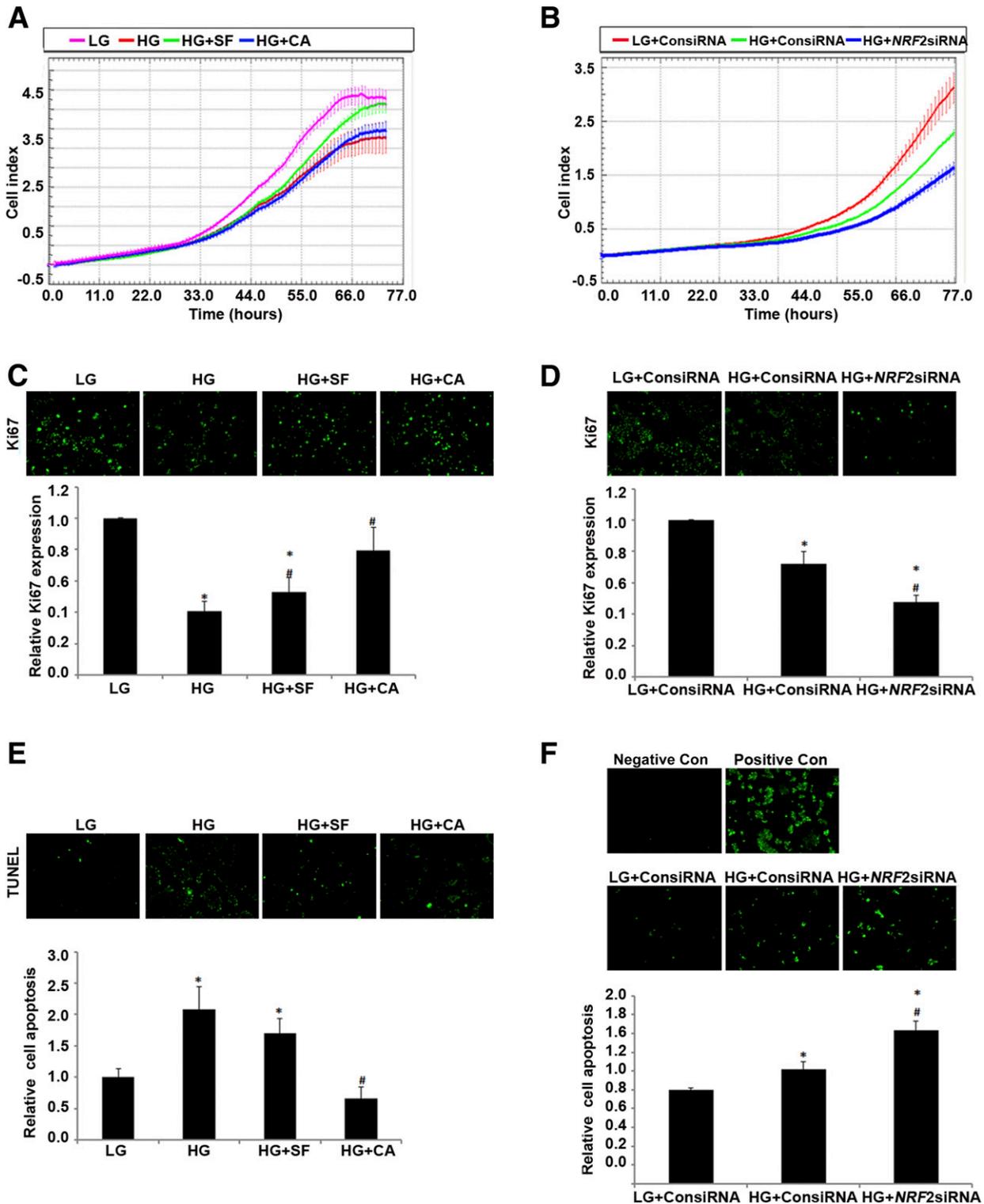


Figure 7—NRF2 activation promotes keratinocyte proliferation and decreases apoptosis. *A* and *B*: Proliferation was assessed as cell growth index. Similarly treated (*A*) or siRNA-transfected (*B*) HaCaT cells were monitored for cell growth in real time. Data are expressed as means \pm SEM ($n = 3$). *C* and *D*: Ki67 immunofluorescence images (top) and quantification of fluorescence intensity (bottom). Similarly treated (*C*) or siRNA-transfected (*D*) HaCaT cells were subjected to immunofluorescence analysis with Ki67 antibodies. The relative Ki67 expression was quantified and analyzed by ANOVA and Tukey post hoc test. Results are expressed as means \pm SEM ($n = 3$). * $P < 0.05$ compared with LG or LG+Con-siRNA; # $P < 0.05$ compared with HG or HG+Con-siRNA. *E* and *F*: In situ cell death assessment by TUNEL assay (top) and quantification (bottom). Similarly treated (*E*) or siRNA-transfected (*F*) HaCaT cells were subjected to TUNEL analysis. For the positive control, cells were treated with 20 $\mu\text{mol/L}$ cisplatin for 24 h. Relative cell apoptosis was quantified and analyzed by ANOVA and Tukey post hoc test. Results are expressed as means \pm SEM ($n = 3$). * $P < 0.05$ compared with LG or LG+Con-siRNA; # $P < 0.05$ compared with HG or HG+Con-siRNA.

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, TGF- β , and migration- and proliferation-related genes) through direct or indirect mechanisms, some of which, as well as 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 nondiabetic 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 with *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 nondiabetic mice, indicating that 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 with 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 pretreatment 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 patients with diabetes) 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 ECM 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 MMPs and a reduction in some growth factors, in particular TGF- β 1, in skin tissue (48–50). Higher MMP9 activity or expression in wound fluid and lower TGF- β 1 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- β 1 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- β 1 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 with diabetes (hyperglycemia, ischemia, hypoxia, advanced glycation end products, etc.) may act as contributing factors. Our results suggest that pharmacological activation of NRF2 signaling positively modulates TGF- β 1 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 infrared thermal imaging 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 patients with diabetes.

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 multifactorial, and the exact mechanisms by which NRF2 may modulate inflammation, granulation tissue, and vascular and neural functions in diabetic conditions remain to be investigated in the future. In addition, a topical NRF2 activator-based formulation applied to the skin of patients with diabetes before ulceration could provide a practical therapeutic intervention.

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