A defect in Nrf2 signaling constitutes a mechanism for cellular stress hypersensitivity in a genetic rat model of type 2 diabetes

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Bitar MS, Al-Mulla F. A defect in Nrf2 signaling constitutes a mechanism for cellular stress hypersensitivity in a genetic rat model of type 2 diabetes. Am J Physiol Endocrinol Metab 301: E1119–E1129, 2011. First published September 6, 2011; doi:10.1152/ajpendo.00047.2011.—Nrf2 regulates the expression and coordinated induction of a battery of antioxidant phase 2 genes that protect cells against the cumulative damaging effects of oxidative stress (OS), a major contributor in the development of chronic diabetic complications. Using cultured dermal fibroblasts from rats with type 2 diabetes (DFs), we investigated the intracellular redox status and the adaptive response to OS, in which Nrf2 plays a central role. Our data confirmed that the generation of superoxide by NADPH oxidase and the mitochondria was enhanced in DFs compared with corresponding controls. This was associated with a decrease in the antioxidant capacity and an increase in the sensitivity of these DFs to hydrogen peroxide-induced necrotic cell death. Nrf2 levels in total cell extracts were diminished, and this abnormality appears to stem from a diabetes-related decrease in Nrf2 protein stability. Endogenous (oligomycin) and exogenous (tert-butylinhydroquinone) induction of OS enhanced the nuclear translocation of Nrf2 and increased the mRNA expression of Nrf2-sensitive genes in control but not DFs. The activity of the GSK-3β/Fyn axis was increased markedly in DFs when compared with the corresponding controls. Chemical inhibition of GSK-3β mitigated the diabetes-related suppression of the OS-induced nuclear accumulation of Nrf2 and the transcriptional activation of the genes downstream of Nrf2. Overall, these findings suggest that an augmentation in GSK-3β/Fyn signaling during diabetes contributes to a deficit in both the cellular redox state and the Nrf2-based adaptive response to OS. Moreover, they may also offer a new perspective in the understanding and treatment of nonhealing diabetic wounds.

NF-E2-related factor 2; glycogen synthetase kinase-3β

TYPE 2 DIABETES (T2D) is characterized by chronic degenerative changes that lead to an increase in morbidity and mortality; thus, the development of effective strategies for their prevention or retardation is critical. The lifelong-sustained effects of oxidative stress (OS), electrophile toxicity, and radiation and inflammation damage appear to contribute in large part to these diabetic complications. Aerobic cells have developed an antioxidant armamentarium for their protection that includes the cumulative damaging effects of oxidative stress (OS), a major contributor in the development of chronic diabetic complications. Using cultured dermal fibroblasts from rats with type 2 diabetes (DFs), we investigated the intracellular redox status and the adaptive response to OS, in which Nrf2 plays a central role. Our data confirmed that the generation of superoxide by NADPH oxidase and the mitochondria was enhanced in DFs compared with corresponding controls. This was associated with a decrease in the antioxidant capacity and an increase in the sensitivity of these DFs to hydrogen peroxide-induced necrotic cell death. Nrf2 levels in total cell extracts were diminished, and this abnormality appears to stem from a diabetes-related decrease in Nrf2 protein stability. Endogenous (oligomycin) and exogenous (tert-butylinhydroquinone) induction of OS enhanced the nuclear translocation of Nrf2 and increased the mRNA expression of Nrf2-sensitive genes in control but not DFs. The activity of the GSK-3β/Fyn axis was increased markedly in DFs when compared with the corresponding controls. Chemical inhibition of GSK-3β mitigated the diabetes-related suppression of the OS-induced nuclear accumulation of Nrf2 and the transcriptional activation of the genes downstream of Nrf2. Overall, these findings suggest that an augmentation in GSK-3β/Fyn signaling during diabetes contributes to a deficit in both the cellular redox state and the Nrf2-based adaptive response to OS. Moreover, they may also offer a new perspective in the understanding and treatment of nonhealing diabetic wounds.

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MATERIALS AND METHODS

Primary dermal fibroblast isolation. Primary rat fibroblasts were derived from dorsal skin biopsies performed on four diabetic Goto-Kakizaki rats (DFs) and four age- (12–14 mo) and sex-matched (female) Wistar control rats (CFs). After sterilization in povidine solution, the rat skin was washed in sterile water and rinsed in 70% ethanol in phosphate buffered saline (PBS). The epidermis and dermis were separated following overnight incubation in 0.25% trypsin-EDTA at 4°C. Samples were washed, diced, and digested for 30 min at 37°C in collagenase type I (250 U/mL) dissolved in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM l-glutamine, and 26 mM HEPES. After collagenase treatment, the cells were dislodged, centrifuged, and resuspended in medium supplemented with 10% fetal bovine serum. The cells were grown under standard conditions, and the medium was changed every 3–4 days. It is noteworthy that control and diabetic fibroblasts from passages 3 to 5 were used for the experiments, and these cells were grown under normoglycemic environment (5.5 mM glucose).

Measurement of superoxide, glutathione, and total cell number. Cells in 96-well plates were washed with Krebs-Ringer buffer and then incubated at 37°C in the presence of 10 μM dihydroethidium (Molecular Probes). Fluorescence readings were taken at Ex = 530 nm and Em = 595 nm over a 30-min period. The determination of GSH was achieved using the fluorescence probe monochlorobimane (MCB, Molecular Probes). Cells were incubated with 100 μM of 4 μM MCB for 20 min at 37°C, and the fluorescence intensity was measured at Ex = 390 nm and Em = 460 nm. Ten microliters of 0.5 mM propidium iodide (Molecular Probes) was added to the well in the presence of 10 μM hydrogen peroxide (HP) for 24 h at 37°C. Cells were assessed for lipid peroxidation using the diphenyl-1-pyrene phosphine (DPPP; Invitrogen) fluorescence-based assay (22). Briefly, cells with or without a stressor [50 μM hydrogen peroxide (HP) for 24 h at 37°C] were incubated with 50 μM DPPP at 37°C in the dark for 30 min, after which they were washed twice with PBS and resaped into 150 μL of PBS. A total of 100 μL of cell solution was added to a 96-well plate. The plate was read for DPPP fluorescence (excitation at 340 nm and emission at 405 nm) in a 96-well fluorescence plate reader. The remaining 50 μL of solution was used for protein determination (BCA-based assay; Pierce).

Cell death assay. Fibroblasts were seeded at 1 × 10⁴ cells/well on a 96-well plate. After a 24-h incubation period, the cells were exposed to various concentrations of HP in serum-free medium at 37°C for 2 h (lactate dehydrogenase (LDH), a marker for necrotic cell death) or 16 h (cell viability and apoptosis assay). Quantification of cell viability and caspase-3-like activity was achieved using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and an Apo-ONE homogenous caspase-3/7 assay (Promega), respectively. The values obtained were normalized to the vehicle-treated controls. To measure the rate of necrotic cell death, a LDH release assay was performed using a CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega). In some experiments, cells were preincubated for 1 h with 2 μg of the ATPase inhibitor oligomycin before exposure to HP.

Drug treatment. Fibroblasts of control and T2D were treated with vehicle or the OS-inducing agents tert-butylhydroquinone (tBHQ; 100 μM, Sigma) or oligomycin (15 μM, Sigma) for 16 h. The cells were processed for total RNA extraction or Western blotting analysis as outlined below. Both of these compounds were dissolved in ethanol with a final concentration of 0.01%. To test for the role of GSK-3β, cells were pretreated with 8 h with lithium chloride (50 mM in DMEM) or TDZD-8 dissolved in DMSO (50 μM), which did not exceed 0.01%.

Subcellular fractionation and Western blotting. Fibroblasts seeded in six-well plates and treated as described above were washed with ice-cold PBS, scraped into PBS, and centrifuged at 1,000 rpm for 5 min. The cell pellet was subjected to subcellular fractionation according to a previously published procedure (38). To make the total cell lysate, the cells were lysed with RIPA buffer supplemented with a protease inhibitor mixture and a phoshatase inhibitor mixture. The protein concentration was determined using BCA protein assay reagents (Pierce). Fifty micrograms of total cell lysate (or cytosolic or nuclear) fractions were resolved on a 10% SDS-polyacrylamide gel, Western blotted, and probed with antibodies specific for Nrf2, Keap1, or Fyn (all from Santa Cruz Biotechnology) or GSK-3β, GSK-3β-phospho-Ser9 (p-GSK3-β), and phospho-GSK-α/β (p-Tyr279/p-Tyr216) (Cell Signaling Technology). The purity of the subcellular fractions was confirmed using the anti-LDH antibody (Chemicon International) and the anti-PCNA antibody (Santa Cruz Biotechnology) for cytosolic and nuclear fractions, respectively. The levels of protein on a Western blot were assessed using Quantity One Image software (Bio-Rad) and normalized against suitable loading controls, including anti-β-actin antibody for total and cytosolic fractions and anti-PCNA antibody for the nuclear fraction.

Protein half-life measurement. Assay of Nrf2 half-life was achieved by treating control and diabetic fibroblasts with 50 μM...
cycloheximide to block protein synthesis. Total cell lysates were collected at different time intervals and subjected to immunoblot with an anti-Nrf2 antibody. The relative intensities of the bands were determined as described above.

Real-time quantitative RT-PCR. Total RNA (1 μg) isolated from cultured fibroblasts using Trizol reagent (Invitrogen) was reverse transcribed for 1 h at 37°C using the High Capacity cDNA Reverse Transcription Kit. Real-time quantitative RT-PCR was performed with the TaqMan Gene Expression Assay and was normalized against 18S RNA using an ABI 7900 Real-time PCR System (Applied Biosystems). Primers and probes were designed by and purchased from Applied Biosystems. Primer efficiency and specificity were verified by amplifying standard dilutions of a probe obtained by pooling all of the samples and by melting curve analysis, respectively. Data were evaluated according to previously established procedures (39).

Nrf2-binding competition assay. Control and diabetic fibroblasts were stimulated with iBHQ (50 μM), and nuclear extracts were used for the determination of Nrf2-binding activity to immobilized ARE using a TransAM Nrf2 kit (Active Motif). Briefly, nuclear extract protein (~5 μg) was incubated in a 96-well plate containing the immobilized consensus Nrf2-binding site. Wells were washed three times, and bound Nrf2 was detected by Nrf2 antibody and secondary antibody conjugated with horseradish peroxidase. The signal was detected spectrophotometrically at 450 nm.

Small-interfering RNA transfection. Expression of Nrf2 was inhibited by small-interfering RNA (siRNA) oligonucleotides. The sequences were designed and synthesized by Qiagen. The best silencing efficiency was obtained by incubating 2.0 × 10⁵ cells/well in a six-well plate with complexes formed by 5 nM siRNA (1 μl) and 9 μl of HiPerfect transfection reagent (Qiagen) dissolved in 90 μl of medium according to the manufacturer’s instructions. The transfection was achieved by adding 0.9 ml of medium to the seeded cells followed by 100 μl of siRNA-HiPerfect complex. Twenty-four hours later, 1 ml of fresh medium was added; 48 h after transfection the cells were exposed to either vehicle or the OS-inducing agents, including HP, oligomycin, or tBHQ. Knockout efficiency was verified by real-time PCR and Western blot.

ELISA assay. Control and diabetic fibroblasts were seeded on a six-well plate at 2.5 × 10⁴/well. After incubation overnight, the cells were treated with or without 50 μM HP in serum/phenol-free medium. After incubation for 16 h, supernatants were collected and analyzed for key inflammatory cytokines, including TNFα, IL-1β, fractalkine, and monocyte chemoattractant protein-1 (MCP-1), using commercially available ELISA kits specific for rats and according to the protocols provided by the manufacturers (R & D Systems and Ray Biotech).

Statistical analysis. All data were expressed as means ± SE. Comparisons between two groups were conducted using Student t-tests. ANOVA was used to compare differences among multiple groups, followed by Tukey post hoc test for significance. A probability value of P ≤ 0.05 was considered statistically significant. All experiments were performed in triplicate on at least three separate occasions.

RESULTS

DFs exhibit a state of heightened OS. OS and antioxidant defense were assessed in fibroblasts derived from control and diabetic rats. Superoxide generation over a 30-min period was 55% higher in DFs relative to corresponding control values (Fig. 1A). This radical is a by-product of mitochondrial respiration and enzymatic oxidases. Accordingly, we examined initially whether the observed elevation in superoxide stemmed from enhanced activity of the nonphagocytic NAD(P)H oxidase. The resulting data showed that VAS2870, a specific NADPH oxidase inhibitor (51), reduced the diabetes-related increase in superoxide by ~31%, thus supporting the partial involvement of NADPH oxidase (Fig. 1A). To more directly assess the involvement of NADPH oxidase in diabetes-related increase in superoxide generation, we determined NADPH-dependent superoxide generation in 28,000-g membrane fractions of control and diabetic fibroblasts using lucigenin chemiluminescence- or the SOD-inhibitable cytochrome c reduction-based assay. DFs produced superoxide at a rate of 6.36 nmol·mg protein⁻¹·min⁻¹, which was significantly higher than corresponding control values (Fig. 1B). Adding various inhibitors of nitric oxide synthetase (e.g., N⁵-nitro-L-arginine methyl ester) or xanthine oxidase (e.g., allopurinol) appears to have not a significant effect on superoxide production (Fig. 2B). However, the specific oxidase inhibitor VAS2870 reduced activity by ~87%, thus confirming that NADPH oxidase activity is indeed upregulated during diabetes (Fig. 1B). Consistent with these data, we also documented that the levels of expression of mRNAs encoding for Nox1 and Nox4 were markedly augmented in DFs compared with corresponding control values (Fig. 1C).

To determine whether mitochondria also contributed to the elevated ROS levels seen in DFs, the mitochondria-targeted superoxide-sensitive fluorophore MitoSOX Red (Molecular Probes) was used. For these studies, parallel measurements using 0.1 μM MTG (Molecular Probes), a probe that selectively stains the mitochondria, were performed to assess total mitochondrial mass. The data derived from these studies demonstrated that mitochondrial superoxide generation when normalized to MTG fluorescence was higher in DFs relative to corresponding control values (Fig. 1D). However, the relative difference in ROS production between CFs and DFs was potentiated to a greater extent by antimiycin A, a selective inhibitor of the complex III of the mitochondria electron transport chain (mETC). Consistent with these data, we also found that protein carbonyl levels, a measurement of ROS-mediated protein oxidation, in the mitochondrial fraction were also elevated as a function of diabetes (nmol/mg protein: CFs, 0.15 ± 0.05; DFs, 0.16 ± 0.05). Overall, the above data indicate that an upregulation in NADPH oxidase expression/activity in connection with a deficit in mETC (a major source of mitochondrial ROS) contributes to the elevation in ROS levels during diabetes.

To monitor whether the diabetes-induced elevation in intracellular levels of ROS may reflect an increase in lipid peroxidation, a marker of accumulative OS, we assessed the lipid peroxide formation of CFs and DFs at baseline and under stressed conditions using the dye DPPP, which intercalates and reacts with lipid hydroperoxides. As shown in Fig. 1E, there was about a 37% enhancement in DPPP fluorescence in unstressed DFs compared with corresponding controls. HP and tert-butylhydroperoxide (tBHP) each elicited an enhancement in lipid peroxidation, which was markedly higher in diabetic than in control cells.

In view of the above data documenting an enhancement in the rate of lipid peroxidation in diabetic fibroblasts, we examined the activity levels of key enzymes in the plasma membrane redox system (PMRS), including CoQ reductase, cytochrome b5 reductase, and NQO1. The PMRS appears to protect against plasma membrane lipid peroxidation triggered by exogenous and endogenous OS. Consistent with the observed
abnormalities in the plasma membrane lipid peroxidation during diabetes, we also found that in this disease state the levels of PMRS-based enzymes, including CoQ-R, cytochrome b5-R, and NQO1, were reduced by 37, 31, and 45%, respectively (Fig. 1F).

GSH represents a key component of the antioxidant defense mechanism, and this system was assessed in our cultured fibroblasts using the fluorescence probe MCB. GSH levels in DFs were reduced by ~40% compared with control counterparts (Fig. 1G). Moreover, we also found that the sensitivity of this ROS scavenging system to various forms of OS, including HP and menadione, was markedly enhanced in DFs (Fig. 1G).

To this end, the aforementioned data are consistent with the notion that an imbalance between oxidant-producing systems and antioxidant defense mechanisms appears to exist during diabetes. This phenomenon may trigger cell damage by oxidizing (as we have shown above) macromolecular structures (lipids, proteins, and DNA) and modifying their functions, leading ultimately to cell death.

**Increased sensitivity of DFs to HP induces necrotic cell death.** A wealth of evidence indicates that chronic oxidative stress, which we clearly confirmed to exist in our DFs, can alter the sensitivity and the mechanism by which a cell dies in response to various stressors (18). Accordingly, in this study, we evaluated the effect of HP, the most common endogenous oxidant, on cell viability and caspase-3-like activities in CFs and DFs. The resulting data showed that exposure of DFs to 37.5, 50, and 75 μM HP for 16 h led to a 17, 55, and 78% loss, respectively (as we have shown above) macromolecular structures (lipids, proteins, and DNA) and modifying their functions, leading ultimately to cell death.

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respectively, in cell viability (Fig. 2B). However, exposure of normal cells to the same concentrations of HP resulted in less marked changes in cellular viability of only 6, 22, and 53%, respectively (Fig. 2B).

Next, the caspase-3-like activity in response to 50 μM HP was also determined; as shown in Fig. 2C, the fold increase in DF caspase-3-like activity (5.4-fold) was less than that of corresponding controls (8.2-fold). In view of these data and the well-known concept that cell death by apoptosis involves a number of energy-dependent steps, including the activation of caspase-3 enzyme (21, 52), we assessed the intracellular level of ATP and found it to be decreased as a function of diabetes (Fig. 2D). This diabetes-related decrease in intracellular free ATP level may stem from an abnormality in the mitochondrial function, as evidenced by the increase in mitochondrial ROS generation (Fig. 1D), elevated mitochondrial protein carbonyl levels, and the decrease in the activity of complex I of the mETC (nmol·mg protein⁻¹·min⁻¹: CFs, 24 ± 3.9; DFs, 13.9 ± 2.2; P ≤ 0.05). To this end, the above data advance the notion that CFs are more resistant than DFs to HP-induced cell death. Moreover, the enhanced cell death seen in DFs appears to be associated with a marked reduction in ATP level, a phenomenon that may favor necrotic over apoptotic cell death. Credence for this proposition is reflected by our data depicted in Fig. 2E showing that, in response to HP, the rate of LDH release into cell culture media, a measure of necrotic cell death, was markedly enhanced in DFs compared with corresponding control values, a finding that was confirmed using the PI intake/FACS-based technique (data not
shown). Further experimentations confirmed that the above enhancement in necrotic cell death during diabetes was accompanied by a marked increase in the expression and rate of release of proinflammatory cytokines, including TNF-α, IL-1β, fractalkine, and MCP-1 (Fig. 2, F and G). The latter state of low-grade inflammation appears to harmonize well with our previous results demonstrating that the transcriptional activity of NF-κB was augmented in fibroblasts and wounded tissues of type 2 diabetes (1a).

Diabetes impairs Nrf2 signaling under basal conditions and in response to OS. To inspect at the molecular level the reasons for the heightened state of OS and the enhanced sensitivity of DFs to HP-induced cell death, we focused on the Nrf2-signaling pathway, a master regulator of the cellular response to OS (34). The data derived from these studies showed that Nrf2 levels in total cellular protein extracts were diminished in DFs relative to CFs (Fig. 3A), an abnormality that appears to be due neither to a reduction in mRNA level (normalized to 18S RNA and expressed as a fold change vs. control: CFs, 1 ± 0.12; DFs, 1.71 ± 0.21; *P* ≤ 0.05) nor to the decrease in mRNA half-life, as shown by an experiment using actinomycin D (10 ng/ml; data not shown). These findings in connection with the confirmed elevation in Keap1 levels (Fig. 3B), a component of an E3 ubiquitin ligase complex that targets Nrf2 for degradation, during diabetes promoted us to assess Nrf2 protein stability using the cycloheximide (CHX) chase-based analysis. In these experiments, we allowed Nrf2 to accumulate to a well-detectable level in CFs/DFs using the MG 132 (5 μM), an inhibitor of the process of 26S proteasome-mediated Nrf2 degradation. One hour later and after extensive washing, the de novo synthesis of Nrf2 was blocked with CHX for the indicated time periods. The data presented in Fig. 3C showed clearly that in

![Fig. 3. Diabetes-induced impairment in the nuclear accumulation of Nrf2 and the induction of phase 2 enzymes following exposure to oxidative stress (OS). A: Nrf2 protein levels in total cell extracts of fibroblasts were measured under basal condition and in response to the OS-inducing agent tert-butyldihydroquinone (tBHQ). B: Western blotting and immunoprecipitation were used to measure total Keap1 level and the degree of binding of Keap1 to Nrf2. C: Nrf2 half-life was assessed using the cycloheximide chase-based assay. D: the nuclear accumulation of Nrf2 in C (lane 1, baseline; lane 2, oligomycin; lane 3, tBHQ) or D (lane 4, baseline; lane 5, oligomycin; lane 6, tBHQ) under basal conditions and in response to OS was assessed using subcellular fractionation and Western blotting. E and F: fibroblast mRNA levels of phase 2 antioxidant enzymes after treatment with tBHQ (E) or oligomycin (F) were evaluated using TaqMan real-time PCR. G: the nuclear extracts described above were also used for the measurement of Nrf2 DNA-binding activity. Values are means ± SE of at least 3 independent determinations. *Significantly different from corresponding control values at *P* ≤ 0.05. **Significantly different from corresponding vehicle-treated values at *P* ≤ 0.05. GSTP, glutathione S-transferase; GR, glutathione reductase; GCLC, glutamate-cysteine ligase catalytic subunit.]
DFs Nrf2 was degraded by the proteasome at a much higher rate than that of CFs. Moreover, we also found in a coimmunoprecipitation analysis that the degree of association of Nrf2 with Keap1 was increased markedly as a function of diabetes (Fig. 3B). Overall, our findings are consistent with the concept that at least in fibroblasts diabetes reduces Nrf2 protein stability, possibly by augmenting the Keap1-dependent signaling pathway.

Next, we studied the nuclear localization of Nrf2 in response to OS induced by oligomycin and tBHQ. Oligomycin-related inhibition of ATPase appeared to produce an overreduction of the mitochondrial quinone pool with a concomitant increase in superoxide generation (16). Similarly, tBHQ may undergo redox cycling either by cellular quinone reductases or through auto-oxidation reactions, resulting in the formation of HP (26). Our data confirmed that, in the nuclei-enriched fraction of CFs, Nrf2 was markedly elevated in response to oligomycin and tBHQ (Fig. 3D). A far smaller increase in the nuclear accumulation of Nrf2 was evident in DFs (Fig. 3D). It is noteworthy that tBHQ elevated the level of Nrf2 in total cell lysates of control and diabetic fibroblasts to about the same extent (Fig. 3A), a finding that is consistent with the concept that a defect in the nuclear localization/export signals for this ARE-transactivating factor may exist during diabetes. Indeed, the data outlined below give credence to the aforementioned contention.

To assess whether the impairment in OS-mediated nuclear translocation of Nrf2 in DFs might affect ARE-responsive genes, we used real-time PCR to measure the mRNA levels of key genes that are regulated by Nrf2. In CFs, the levels of expression of catalase, glutathione S-transferase P1 (GSTP1), glutamate-cysteine ligase catalytic subunit (GCLC), and NQO1 in response to tBHQ were increased by 8.8, 6.3, 5.7, and 4.8-fold, respectively (Fig. 3E). However, in DFs, enhancement of the aforementioned Nrf2-dependent transcripts was greatly reduced; indeed, the percent increase was in the range of 32–78% (Fig. 3E). We did not notice any changes in the levels of either Cu/Zn SOD or Mn-SOD. Oligomycin treatment also caused an increase in the expression of MnSOD, catalase, GSTP1, and GCLC but not of NQO1 in CFs (Fig. 3F). This phenomenon was markedly suppressed as a function of diabetes (Fig. 3F). These results corroborate very well with the data depicted in Fig. 3G showing that an oligomycin- or tBHQ-induced increase in the transcriptional activity of Nrf2, assessed using an immobilized oligonucleotide containing the ARE consensus binding site, was markedly suppressed as a function of diabetes.

Overall, our results suggest that a defect in the Nrf2-signaling pathway may contribute, at least in part, to the increased sensitivity of DFs to OS-induced necrotic inflammation and cell death. A credence for the aforementioned proposition is the previous studies showing that 1) the activation of Nrf2 protects against ethanol- or ischemic/reperfusion-induced cell death (12, 15), 2) the degree of increase in liver necrosis following bromopropane treatment was enhanced markedly in Nrf2-null mice compared with corresponding wild-type mice (35), and 3) knockout of Nrf2 in control fibroblasts recapitulated the diabetic cell phenotype (please see below).

Knockout of Nrf2 in control fibroblasts recapitulated the diabetic cell phenotype. To confirm the participation of Nrf2 in the increased sensitivity of DFs to HP-induced necrotic cell death, we performed RNA-silencing experiments. CFs were transfected with a silencing RNA (sirNA) sequence directed against Nrf2 mRNA, and the effectiveness of this strategy was evaluated using a real-time PCR-based technique. The data showed a significant reduction in Nrf2 mRNA at 24 h following transfection, and this effect continued for ≤48 h (Fig. 2A). A parallel experiment was conducted with a commercially available siRNA directed against GAPDH to control for transfection and silencing efficiency. A marked decrease in the rate of GAPDH mRNA expression was evident at 24 and 48 h postinfection (data not shown). As a control for the silencing specificity and for off-target effects, cells were also transfected with a commercially available nonsilencing siRNA-like sequence, which does not recognize any eukaryotic sequence (Fig. 2A). Following this confirmation step, we exposed the Nrf2-knockout fibroblasts and their control counterparts to various concentrations of HP. The data revealed that the degree of loss in cell viability as well as the rate of release of LDH at each of the HP concentrations were significantly higher in Nrf2-knockout fibroblasts relative to corresponding controls (Fig. 2, B and E). This phenomenon was associated with a greater accumulation of several inflammatory cytokines following HP treatment (Fig. 2, F and G). Further experiments also showed that Nrf2-knockout fibroblasts, much like DFs, exhibited a significant decrease in the various Nrf2-related genes both at the basal level and in response to tBHQ or oligomycin (Fig. 3, E and F).

Enhanced GSK-3β activity and increased expression of Fyn in DFs contributed to reduced Nrf2 nuclear accumulation in response to OS. Recent data have shown that GSK-3β inhibits the nuclear accumulation of Nrf2 in response to OS via a Fyn-dependent mechanism (25). We examined this sequence of events in DFs because in these cells the nuclear accumulation of Nrf2 was markedly diminished. Since the activity of GSK-3β is regulated negatively by the phosphorylation of serine 9 (p-GSK-3β-Ser9) and positively by the phosphorylation of tyrosine 216 (p-GSK-3β-Tyr216) (19), we compared the expression/phosphorylation status of GSK-3β between the control and diabetic fibroblasts. As shown in Fig. 4A, the degree of GSK-3β inactivation, as determined by the level of phospho-Ser-9 GSK-3β, was markedly reduced in DFs compared with corresponding control values. In contrast, an increase in the level of p-GSK-3β-Tyr216 was evident in these cells (Fig. 4A). This diabetes-related enhancement in the GSK-3β activity was associated with a significant increase in the level of expression of Fyn kinase (Fig. 4A), a downstream target of GSK-3β and an important enzyme in the control of nuclear export and degradation of Nrf2 (27).

Pharmacological and siRNA-mediated downregulation of GSK-3β activity ameliorated the defect in Nrf2-dependent signaling during diabetes. Next, we analyzed whether GSK-3β inhibitors had any effect on the nuclear accumulation of Nrf2 in DFs. The most established inhibitors of GSK-3β, including lithium and thiadiazolidinone TDZD-8, were used, with the resulting data confirming that both the basal and inducible levels of nuclear Nrf2 were partially normalized in response to the aforementioned treatment (Fig. 4B, only data for lithium shown). Consistent with these data, we also confirmed that the pharmacological inhibition of GSK-3β also partially restored the transcriptional activity of Nrf2 as well as the sensitivity of Nrf2-dependent genes to the OS-inducing agents, including tBHQ and oligomycin (Fig. 4, C–E).
To obtain additional evidence for the importance of GSK-3β in diabetes-induced impairment in the Nrf2-signaling pathway and to avoid potential artifacts induced by inhibitory drugs, the level of GSK-3β was downregulated using siRNA. Silencing of GSK-3β in DFs recapitulated most of the changes seen with lithium treatment, including the increase in Nrf2 nuclear accumulation, Nrf2 transcriptional activity, and the expression of Nrf2-dependent genes (data not shown).

Impaired Nrf2 signaling in a 7-day diabetic wound. To determine whether the defect in the Nrf2-signaling pathway in DFs is a phenomenon seen only in cell culture, we assessed the basal and tBHQ-induced nuclear accumulation of Nrf2 in in vivo 7-day control and diabetic wounds. The resulting data confirmed that there was a significant decrease in the basal and OS-mediated accumulation of Nrf2 in the nuclei of 7-day diabetic wounds compared with control wounds (unpublished observations). Consistent with these data, we also found that the level of expression of Fyn and the activity of GSK-3β, as reflected by the reduced level of p-GSK-3β, were markedly elevated in the diabetic wounds.

Overall, our data support the notion that an augmentation in GSK-3β/Fyn signaling in T2D reduces the nuclear accumulation of Nrf2 and its downstream effectors in wounds and cultured dermal fibroblasts under both basal conditions and in response to OS. It will be of interest to learn more about how the unusual endocrine environment of the diabetic rat alters Nrf2 expression and how these cellular properties are maintained, possibly by epigenetic mechanisms, through several passages in culture.

DISCUSSION

The current findings illuminate a novel concept regarding the impaired adaptive response of cultured DFs to endogenous and exogenous OS, in which the Nrf2/GSK-3β/Fyn signaling pathway plays a central role. In addition, our data also provide a rational interpretation for the hypersensitivity of DFs to HP-induced cell death. First, we confirmed that a heightened state of OS, as exemplified by the increase in the intracellular levels of ROS and the decrease in the antioxidant defense mechanism, is a characteristic feature of DFs. Second, the suppression of fibroblast GSK-3β expression or activity using lithium or TDZD-8 ameliorated the diabetes-related deficit in Nrf2 nuclear accumulation along with producing a significant increase in the levels of Nrf2-dependent phase 2 antioxidant enzymes. Third, a knockout of Nrf2 in CFs mimicked in many respects the phenotypic features of DFs.

An increase in the key indices of inflammation (e.g., TNFα, IL-1β, MCP-1, fractalkine) and OS (e.g., GSSG/GSH ratio, superoxide generation, and lipid peroxidation) was evident in DFs. These findings prompted us to hypothesize that diabetic cells may have developed a strategy that enables them to survive under conditions of chronic OS and low-grade inflammation. To address this premise, we examined the Keap1/Nrf2/ARE pathway, which is known to be involved in immune and...
inflammatory processes in addition to its critical role in OS and the induction of phase 2 enzymes (54). Our data revealed a significant diminution in the nuclear accumulation of Nrf2 and a marked increase in the cellular content of Keap1 in fibroblasts and skin wounds of T2D. Consistent with these data, the transcriptional activity of Nrf2 as well as the levels of expression of several downstream effectors of Nrf2, including GR, GCLC, and NQO1, were also decreased in diabetes. To demonstrate the decreases in GR, GCLC, and NQO1, we performed activity assays, which correlated well with the changes seen in mRNA levels of these Nrf2-dependent antioxidant enzymes (data shown only for NQO1). In view of our data, it is possible to suggest that diabetic cells or wounds not only face states of chronic inflammation and OS but also show impaired adaptive responses to these stresses. This notion was supported by an experiment where fibroblasts derived from control and diabetic animals were subjected to endogenous and exogenous OS using oligomycin and tBHQ, respectively. The data confirmed that control fibroblasts or wounds showed an adaptive induction in the Nrf2-dependent signaling pathway, a response that was severely impaired in diabetes. The biological relevance of these findings was illuminated by the observation that DFs were more susceptible to HP-induced cell death than corresponding controls. Moreover, this deficit in Nrf2 activation may also contribute to the observed impairment in the induction of phase 2 antioxidant enzymes and to the heightened states of OS and inflammation in cells and possibly wounds of T2D. Interestingly, knocking down Nrf2 in CFs recapitulated most of the aforementioned diabetic features. This harmonizes well with previously published results confirming that Nrf2-knockout mice showed prolonged inflammation during cutaneous wound healing (10), increased OS, and impaired liver regeneration (7). Moreover, Nrf2-deficient animals also appeared to exhibit a high susceptibility to a variety of oxidative stressors, including glucose-induced oxidative cardiomyocyte damage, acetaminophen toxicity, and hyperoxic lung injury (28, 46). Very recently, hepatocyte-specific conditional Keap1-knockout and Nrf2 wild-type mice, but not Nrf2-knockout mice, pretreated with CDDO-Im (an activator of Nrf2) were found to be highly resistant to concanavalin A-mediated inflammatory liver injury (42). Finally, the anti-inflammatory potencies of a number of chemical classes appear to correlate with the potencies of these agents to induce phase 2 enzymes, including NQO1, cytochrome b5 reductase, and CoQ, also protects cells against lipid peroxidation chain reactions (23) and maintains cellular redox status via NAD(P)H recycling (6). Nrf2 regulates GSH homeostasis by affecting GCLC and NQO1/cytochrome b5 reductase mRNA and enzyme activity in diabetic fibroblasts and possibly wounds. Moreover, the aforementioned proposition also provides a rational explanation for the shift toward oxidation in the glutathione redox state [control: 5.7% glutathione disulfide (GSSG)/94.3% GSH vs. diabetic: 37% GSSG/63% GSH] and to the decreased activity of PMRS and enhanced susceptibility of diabetic cells/tissues to lipid peroxidation and lethal oxidative damage.

Third, we must investigate the sequence of events responsible for the diabetes-related deficit in the OS-induced nuclear accumulation of Nrf2. Several lines of evidence indicate cross-talk between the Nrf2 and phosphatidylinositol 3-kinase (PI3K) pathways. Astrocytes treated with PI3K inhibitors showed a concentration-dependent decrease in GSH synthesis and Nrf2 activity (53). A similar effect was also documented by transfection with a siRNA against p85α (53). Finally, a functional PI3K/Akt-dependent pathway is required for a number of Nrf2-inducing agents, including hemin, tBHQ, and peroxynitrite (30, 40).

PI3K generates signaling lipids that recruit and activate Akt via protein kinases, including 3′-phosphoinositide-dependent kinase 1, Thr308, and Ser473 (11). Activated Akt phosphorylates various downstream substrates that are essential in the regulation of cell cycle progression and survival (29). Among these is GSK-3β, which gets activated or inactivated by HP- or Akt-mediated phosphorylation at Tyr172 or Ser9, respectively (25). This serine/threonine protein kinase has been shown to act upstream of Fyn kinase in regulating the nuclear export and degradation of Nrf2 (25) in addition to blocking the xenobiotic and antioxidant cell responses (49). Recent data from our...
laboratory confirmed that PI3K/Akt signaling was impaired in cultured DFs (unpublished observations). Moreover, we also showed that 7-day cutaneous wound sensitivity to IGF-I, a well-known activator of the PI3K/Akt pathway, was diminished in diabetes (unpublished observations). These findings, together with those garnered from the literature regarding the negative effects of Keap1 on the level of Nrf2, give credence to the notion that the dysregulated activation of Nrf2 and its downstream effectors of phase 2 antioxidant enzymes within the diabetic milieu may stem from an enhancement in the activity of the GSK-3β/Fyn-signaling pathway and/or a marked increase in the level of Keap1. Consistent with this proposition are the current results showing that the decrease in the nuclear accumulation of Nrf2 during diabetes is associated with a marked increase in GSK-3β activity and its target Fyn kinase and, indeed more intriguingly, that we can partially normalize the diabetes-related impairment in the Nrf2/ARE/phase 2 enzymes using well-established inhibitors of GSK-3β activity, including lithium and TDZD-8. Further experiments are under consideration in our laboratory to more clearly define the mechanism of Nrf2 inhibition by GSK-3β/Fyn or Keap1 in DFs and wounds.

Overall, our data favor the notion that the diabetes-induced enhancement in GSK-3β/Fyn-dependent signaling and/or Keap1 accounts, at least in part, for the marked reduction in the nuclear accumulation of Nrf2/transcriptional activity in response to OS. The functional consequences of this phenomenon are best illuminated by the decrease in the antioxidant defense mechanisms of DFs and their subsequent supersensitivity to OS. Moreover, the above findings may also provide an opportunity for the development of interventional strategies to restore normal redox control and to protect against OS in diabetes, thereby preventing some of the diabetes-related complications such as the chronic nonhealing wounds.

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DISCLOSURES

The authors declare no financial or other conflicts of interest.

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