Diabetic Downregulation of Nrf2 Activity via ERK Contributes to Oxidative Stress–Induced Insulin Resistance in Cardiac Cells In Vitro and In Vivo

Yi Tan,[1,2] Tomonaga Ichikawa,[3] Jinqing Li,[3] Qiusheng Si,[4] Huaitao Yang,[4] Xiangbai Chen,[4] Curtis S. Goldblatt,[4] Colin J. Meyer,[5] Xiaokun Li,[1] Lu Cai,[1,2] and Taixing Cui[1,3]

OBJECTIVE—Oxidative stress is implicated in cardiac insulin resistance, a critical risk factor for cardiac failure, but the direct evidence remains missing. This study explored a causal link between oxidative stress and insulin resistance with a focus on a regulatory role of redox sensitive transcription factor NF-E2–related factor 2 (Nrf2) in the cardiac cells in vitro and in vivo.

RESEARCH DESIGN AND METHODS—Chronic treatment of HL-1 adult cardiomyocyte with hydrogen peroxide led to insulin resistance, reflected by a significant suppression of the insulin-induced glucose uptake. This was associated with an exaggerated phosphorylation of extracellular signal–related kinase (ERK). Although U0126, an ERK inhibitor, enhanced insulin sensitivity and attenuated oxidative stress–induced insulin resistance, LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K), worsened the insulin resistance. Moreover, insulin increased Nrf2 transcriptional activity, which was blocked by LY294002 but enhanced by U0126. Forced activation of Nrf2 by adenoviral overexpression of Nrf2 inhibited the increased ERK activity and recovered the blunted insulin sensitivity on glucose uptake in cardiomyocytes that were chronically treated with H_2O_2. In the hearts of streptozotocin-induced diabetic mice and diabetic patients Nrf2 expression significantly decreased along with significant increases in 3-nitrotyrosine accumulation and ERK phosphorylation, whereas these pathogenic changes were not observed in the heart of diabetic mice with cardiac-specific overexpression of a potent antioxidant metallothionein. Upregulation of Nrf2 by its activator, Dh404, in cardiomyocytes in vitro and in vivo prevented hydrogen peroxide– and diabetes-induced ERK activation and insulin-signaling downregulation.

CONCLUSIONS—ERK-mediated suppression of Nrf2 activity leads to the oxidative stress–induced insulin resistance in adult cardiomyocytes and downregulated glucose utilization in the diabetic heart. ***Diabetes*** **60:625–633, 2011**

From the [1]Chinese-American Research Institute for Diabetic Complications, Wenzhou Medical College, Wenzhou, Zhejiang, China; the [2]Department of Pediatrics, University of Louisville, Louisville, Kentucky; the [3]Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, South Carolina; the [4]Department of Pathology, Memorial Medical Center, Johnstown, Pennsylvania; and the [5]Reata Pharmaceuticals, Irving, Texas.

Corresponding authors: Taixing Cui, taixing.cui@uscmed.sc.edu, and Lu Cai, L0cai001@louisville.edu.

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Y.T. and T.I. contributed equally to this work.

Insulin resistance usually refers to a defect in the ability of insulin to stimulate glucose uptake. Importantly, insulin resistance is not only a characteristic feature of type 2 diabetes but also implicated in the heart of type 1 diabetes (1,2). The severity of insulin resistance independently predicts mortality in patients with heart failure (3,4). At a molecular level, insulin resistance is characterized by impairment of the insulin-induced activation of insulin receptor substrate (IRS)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, the major player of the metabolic action of insulin, leading to suppression of the insulin-induced glucose uptake in the insulin-sensitive organs, including the heart (3,4). Indeed, reduced IRS-1 associated PI3K activity in skeletal muscle has been demonstrated to contribute to whole body insulin resistance in diabetes (5). These results reveal a unique feature of cardiac insulin resistance that might play a crucial role in the pathogenesis of diabetic cardiomyopathy.

Although a pivotal role of insulin resistance in cardiac dysfunction appears to be recognized, the mechanisms contributing to insulin resistance are poorly understood. Notably, increasing evidence has suggested that oxidative stress plays a causal role in the cardiac complications of insulin resistance, and the overgenerated reactive oxygen species (ROS) or reactive nitrogen species (RNS) and insulin resistance may be coconspirators in cardiac dysfunction, each capable of triggering or worsening the other (6–8). However, a direct, clear, and causal relationship between oxidative stress and insulin resistance in the heart is not yet established.

Cells have evolved endogenous defense mechanisms against sustained oxidative stress such as the redox sensitive transcription factor NF-E2–related factor 2 (Nrf2), which regulates antioxidant response element (ARE/EpRE)-mediated expression of detoxifying and antioxidant enzymes and the cystine/glutamate transporter involved in glutathione biosynthesis (9). Diminished Nrf2/ARE activity contributes to increased oxidative stress and mitochondrial dysfunction in the vasculature, leading to endothelial dysfunction, insulin resistance, and abnormal angiogenesis observed in diabetes (10). Recently we have demonstrated the critical role of Nrf2 expression in protecting the cardiac cells from oxidative damage and death caused by high levels of glucose (11).

Herein, we demonstrate that oxidative stress directly induces insulin resistance in cardiomyocytes via exaggerating extracellular signal–related kinase (ERK) activity in vitro. In the heart of streptozotocin (STZ)-induced type 1 diabetic

mice, cardiac expression of Nrf2 was significantly depressed at the late stage of diabetes, the time period at which the heart showed a significant decrease in glucose metabolism along with the development of significant diabetic cardiomyopathy (12–14). Depressed expression of cardiac Nrf2 was associated with significant increases in nitrosative damage and phosphorylation of ERK, all of which were prevented in the hearts of diabetic mice with cardiac overexpression of a potent antioxidant metallothionein (MT). In addition, upregulation of cardiac Nrf2 by its activator dihydro-CDDO-trifluoroethyl amide (Dh404) significantly prevented diabetes-induced nitrosative damage, ERK activation, and insulin signaling downregulation. These findings suggest that oxidative stress–depressed expression of cardiac Nrf2 is associated with cardiac activation ERK and downregulation of glucose metabolism. Therefore, Nrf2, a master transcriptional factor of antioxidative defense system (15), may be a novel negative regulator of oxidative stress–mediated insulin resistance in cardiomyocytes and the heart.

RESEARCH DESIGN AND METHODS

Cell culture and adenoviral infection. HL-1 cells were obtained from Dr. Claycomb (Louisiana State University Health Science Center, New Orleans, LA), who first established and characterized the cell line derived from adult murine atrial cardiomyocyte tumor lineage, and cultured in Claycomb Medium (Sigma-Aldrich) (16). Cells were infected with adenovirus of β-galactosidase (Ad-βGal) or murine Nrf2 (Ad-Nrf2) as previously described (15).

Glucose uptake assay. Cells were incubated in Krebs-Ringer-HEPES buffer (15 mmol/L HEPES [pH 7.4], 105 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 1 mmol/L KH_2PO_4, 1.4 mmol/L $MgSO_4$, and 10 mmol/L $NaHCO_3$) for 30 min. Cells next were incubated with insulin (100 nmol/L) in Krebs-Ringer-HEPES buffer for 30 min and then added to 2-deoxy-D-glucose (0.2 mmol/L) and 2-deoxy-D-[^{3}H] glucose (2-DG) (1 μCi/mL, MP Biomedicals) for an additional 10 min. The cells were washed three times with ice-cold PBS, solubilized in 0.5 mol/L NaOH, and neutralized with 0.5 mol/L HCl, and the radioactivity in aliquots from cell extracts was measured using a liquid scintillation counter. Protein concentration of the cell extracts was measured using a protein assay kit (Bio-Rad). Deoxyglucose uptake was expressed as counts per minute per microgram of protein.

Nrf2 transcriptional reporter assay. Cells were transfected with Nrf2 transcription reporter gene *ARE-luc* (firefly luciferase) and internal control *pRL-TK-luc* (renilla luciferase) plasmids, and Nrf2 transcriptional activity was quantified by measuring luciferase activities as previously described (17).

Diabetes models and drug administration. MT-overexpressing transgenic (MT-TG) mice were produced from the FVB mice that have been well characterized (12–14). Both 8- to 10-week-old MT-TG positive mice (heterozygotes) and negative littermates (wild type [WT]) were kept in the same cages with free access to rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care. STZ (Sigma, St. Louis, MO) was dissolved in sodium citrate buffer (pH 4.5). Male mice were given an intraperitoneal injection of STZ at 40 mg/kg body wt daily for five days. Whole blood glucose obtained from the murine tail vein was detected using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA) five days after the last STZ injection. STZ-injected mice with glucose levels >12 mmol/L were considered diabetic, and mice serving as controls were given the same volume of sodium citrate (12–14).

Two animal studies were performed. The first study was to investigate the diabetic effect on cardiac Nrf2 expression along with nitrosative damage, measured by 3-nitrotyrosine (3-NT) and ERK expression, using MT-TG and WT diabetic mice at 2 and 5 months after diabetes onset. The second study was to investigate the preventive effect of Nrf2 activator Dh404 (provided by Reata Pharmaceuticals) on cardiac Nrf2 expression and 3-NT as well as insulin signaling targets including phosphorylation of Akt and glycogen synthase kinase-3β (GSK-3β) at 2 weeks after diabetes onset. For Dh404 treatment, diabetic and age-matched nondiabetic mice were administrated with Dh404 (10 mg/kg body wt) or vehicle (sesame oil; Sigma) every other day by gavage. Two weeks later, 6 h after the last dose administration, mice were killed and heart tissues were collected for the study.

Immunochemical staining and immunoblot analysis. Staining and Western blot analysis for Nrf2, total and phosphor-ERK1/2, total and phosphor-Akt, total and phosphor-GSK-3β, and 3-NT were performed as previously described (9,12–14). Briefly, heart tissues were homogenized in lysis buffer using homogenizer. Tissue proteins were collected by centrifuging at 12,000*g* at 4°C in a Beckman GS-6R centrifuge for 15 min. The protein concentration was measured by Bradford assay. The sample, diluted in loading buffer and heated at 95°C for 5 min, was then subjected to electrophoresis on 10% SDS-PAGE gel at 120 V. After electrophoresis of the gel and transfer of the proteins to nitrocellulose membrane, the membranes were rinsed briefly in Tris-buffered saline, blocked in blocking buffer (5% milk and 0.5% BSA) for 1 h, and washed three times with Tris-buffered saline containing 0.05% Tween 20. The membranes were incubated with different primary antibodies at a dilution of 1:1,000 for 2 h and then washed and reacted with secondary horseradish peroxidase–conjugated antibody for 1 h. Antigen-antibody complexes were then visualized using an ECL kit (Amersham, Piscataway, NJ).

Human heart specimens. Tissue sections of left ventricles were prepared from autopsy heart specimens of humans with or without a disease history or diabetes. All diabetic males had histories of hypertension and cardiac dysfunction (Supplementary Table 1). This study was approved by the Institutional Review Board for human subject research at the University of South Carolina School of Medicine and the Memorial Medical Center of Johnstown.

Statistical analysis. Values are expressed as means ± SD. The data were analyzed using ANOVA with the Newman-Keuls test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Oxidative stress induces insulin resistance via overactivation of ERK in adult cardiomyocytes. Chronic treatment with H_2O_2 has been demonstrated to induce oxidative stress leading to insulin resistance in various cell types (18); therefore, we tested whether such a treatment induces insulin resistance in HL-1 adult cardiomyocytes. Consistent with a previous report (19), insulin stimulated 20–30% increases in glucose uptake in HL-1 cells (Fig. 1*A*), and treatment with H_2O_2 at a nontoxic

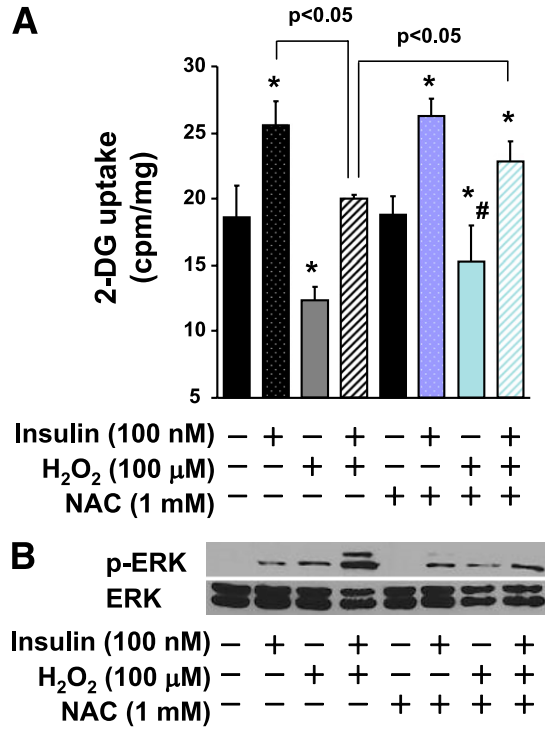

FIG. 1. H_2O_2-induced insulin resistance in HL-1 cells. Cells were pretreated with H_2O_2 (100 μmol/L) with or without NAC (1 mmol/L) in 0.1% FBS medium in the absence of norepinephrine for 16 h and then subjected to basal or insulin (100 nmol/L for 10 min)-stimulated 2-DG uptake assay (*A*), followed by Western blotting of phosphorylated and total ERK (*B*). Results are representatives of 3 separated experiments ($n = 4$). *$P < 0.05$ vs. control (H_2O_2− and insulin−), #$P < 0.05$ vs. H_2O_2 (+) without NAC; otherwise statistical difference was indicated. (A high-quality color representation of this figure is available in the online issue.)

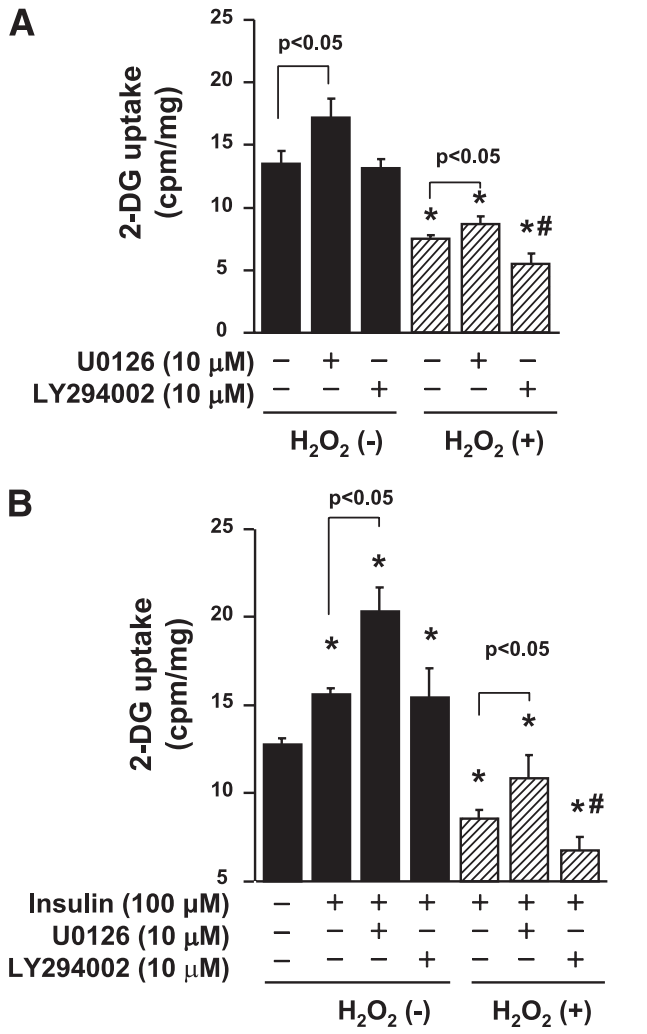

FIG. 2. Effect of inhibitors of mitogen-activated protein kinase kinase (MEK) and PI3K on basal or insulin-induced glucose uptake in HL-1 cells with or without oxidative stress. Cells were pretreated with or without H_2O_2 (100 μmol/L) as in Fig. 1, with absence (*A*) and presence (*B*) of 100 nmol/L insulin pretreatment for 10 min. Cells were treated with or without U0126 (10 μmol/L) or LY294002 (10 μmol/L) as indicated for 30 min and then subjected to 2-DG uptake assay. **P* < 0.05 vs. control (−), #*P* < 0.05 vs. insulin (+) plus H_2O_2 (+); *n* = 4.

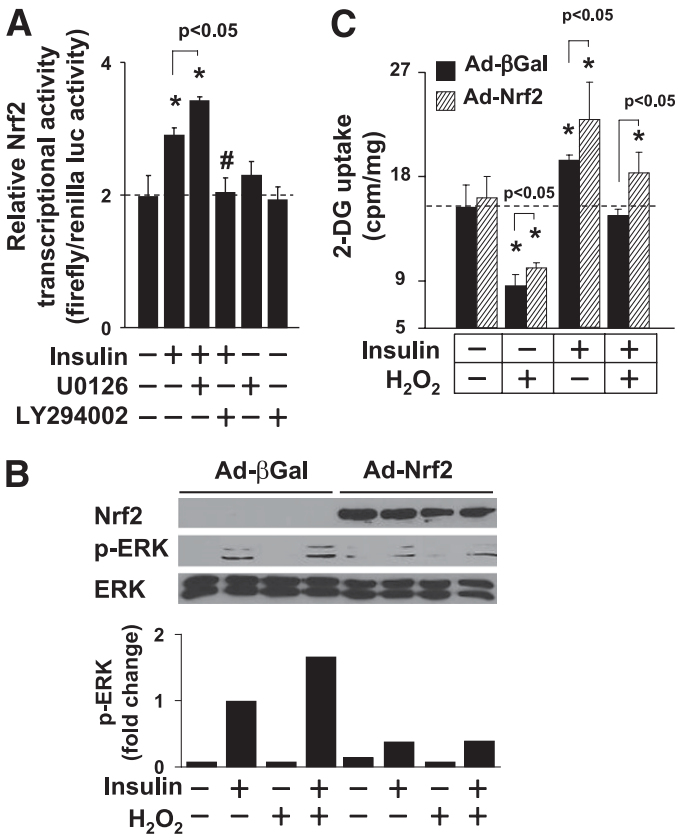

FIG. 3. A negative regulation of Nrf2 by ERK worsens insulin sensitivity in HL-1 cells. *A*: Insulin activates Nrf2 via a negative cross-talk between PI3K and ERK. Cells were transfected with *ARE-luc* and *pRL-TK-luc* in Opti-MEM (Invitrogen) for 6 h and then changed with 0.1% FBS medium in the absence of norepinephrine for 48 h. The cells were next stimulated with 100 nmol/L of insulin, 10 μmol/L of U0126, and 10 μmol/L of LY294002 as indicated for 12 h. Nrf2 transcriptional activity was measured by a dual luciferase assay kit (Promega). **P* < 0.05 vs. control (−), #*P* < 0.05 vs. insulin (+); *n* = 4. *B*: Effect of overexpression of Nrf2 on insulin sensitivity in HL-1 cells. Cells infected with Ad-*βGal* (20 multiplicity of infection [MOI]) and Ad-Nrf2 (20 MOI) were pretreated with H_2O_2 (100 μmol/L) as in Fig. 1 and subjected to 2-DG uptake assay. **P* < 0.05 vs. control (−), *n* = 4. *C*: Infected cells were pretreated as in Fig. 1 and stimulated with or without insulin (100 nmol/L) for 10 min. **P* < 0.05 vs. control (H_2O_2− and insulin−); otherwise statistical difference was indicated.

dose of 100 μmol/L for 16 h suppressed not only the basal but also the insulin-induced glucose uptake (Fig. 1*A*). Pretreatment of *N*-acetyl-cysteine (NAC), a ROS scavenger, partially reversed H_2O_2-induced inhibition of the basal and insulin-induced glucose uptake (Fig. 1*A*), suggesting that oxidative stress directly causes insulin resistance in adult cardiomyocytes.

Potential link of ERK1/2 with oxidative stress and insulin resistance in cardiomyocytes has been documented (19). Accordingly, we examined the effect of chronic treatment of H_2O_2 on ERK1/2 phosphorylation in HL-1 with and without insulin stimulation. As shown in Fig. 1*B*, acute stimulation of insulin (30 min) or chronic treatment of H_2O_2 (16 h) increased ERK phosphorylation; however, the insulin-induced ERK activity was significantly enhanced by H_2O_2. NAC did not affect the insulin-induced ERK activity but suppressed ERK activity induced by H_2O_2 per se or H_2O_2 with insulin (Fig. 1*B*). The reservation of H_2O_2-induced insulin resistance (Fig. 1*A*) and suppression of H_2O_2-mediated ERK activity (Fig. 1*B*) by NAC reflects a possible role of ERK in oxidative stress–induced insulin resistance in cardiomyocytes.

ERK negatively regulates insulin-induced glucose uptake in adult cardiomyocytes. We next examined the role of ERK and PI3K in regulating insulin-mediated glucose uptake in HL-1 cells. In control cells, addition of U0126, an ERK inhibitor, enhanced the basal (Fig. 2*A*) and insulin-induced glucose uptake (Fig. 2*B*), respectively; surprisingly, LY294002, a PI3K inhibitor, had no effect (Fig. 2*A* and *B*). In the cells with chronic H_2O_2 treatment, U0126 was able to partially reverse, but LY294002 worsened, the H_2O_2-mediated inhibition of glucose uptake (Fig. 2*A*) and insulin resistance (Fig. 2*B*). These results support the above hypothesis that ERK as a negative regulator of insulin-mediated glucose uptake plays a mediator role in oxidative stress–induced insulin resistance in adult cardiomyocytes.

Positive regulation of insulin by Nrf2 to stimulate glucose uptake in adult cardiomyocytes. Because we have demonstrated that Nrf2 negatively regulates oxidative

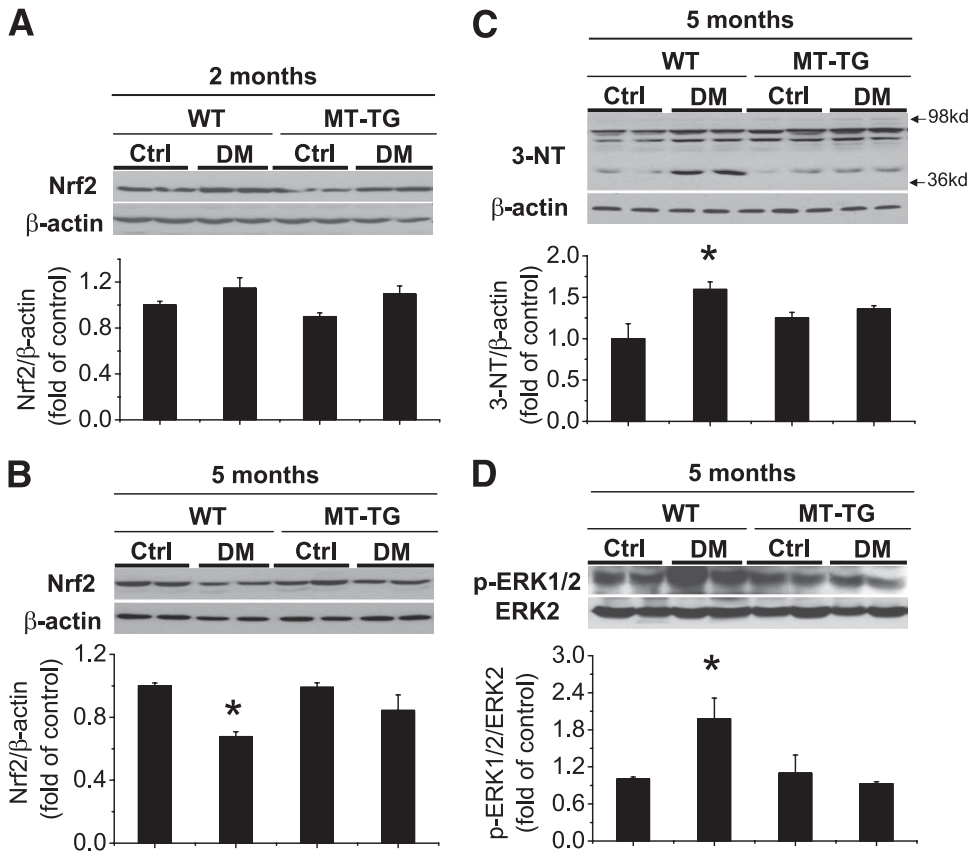

FIG. 4. Cardiac Nrf2 expression in the mice with and without cardiac-specific MT overexpression. Diabetes was induced by multiple low doses of STZ in mice with cardiac-specific MT overexpression (MT-TG) and littermate WT mice. *A*: Cardiac Nrf2 expression at 2 months after the onset of diabetes was detected by Western blot (WT, *n* = 6 for control [Ctrl], *n* = 8 for diabetes mellitus [DM]; MT-TG, *n* = 7 for control, *n* = 8 for DM). At 5 months after the onset of diabetes, cardiac Nrf2 expression (*B*); nitrosative damage, measured by 3-NT (*C*); and ERK phosphorylation (*D*) were detected by Western blot, respectively (WT, *n* = 7 for control, *n* = 8 for DM; MT-TG, *n* = 6 for control, *n* = 7 for DM). β-Actin or total ERK was used as loading control. **P* < 0.05 vs. control.

stress in the heart (15), we then determined whether Nrf2 plays a role in regulating the oxidative stress–induced insulin resistance in HL-1 cells. Nrf2 transcription reporter assay showed that insulin activated Nrf2, which was enhanced by U0126, whereas blocked by LY294002 (Fig. 3*A*), suggesting that the PI3K-mediated Nrf2 activation is negatively regulated by ERK in insulin-stimulated HL-1 cells. To further test whether Nrf2 plays a critical role in regulating insulin-mediated glucose uptake, especially under an oxidative status, we examined effects of forced activation of Nrf2 on the oxidative stress–induced activation of ERK as well as the subsequent insulin resistance in adult cardiomyocytes. As shown in Fig. 3*B*, a forced activation of Nrf2 by adenoviral overexpression of Nrf2 significantly suppressed H_2O_2-induced ERK activity in HL-1 cells with the presence of insulin. Most importantly, the forced activation of Nrf2 enhanced the insulin-mediated glucose uptake in non–H_2O_2-treated cells, relative to the control of Ad-βGal overexpression and completely recovered insulin sensitivity to stimulate glucose uptake in H_2O_2-treated cells (Fig. 3*C*).

Cardiac Nrf2 expression was downregulated in diabetic animals and patients. To explore a pathological relevance of Nrf2 in diabetic hearts, we examined cardiac Nrf2 expression in STZ-induced diabetic mice at 2 and 5 months after diabetes onset. The glucose levels of these diabetic mice were 25.76 ± 2.14 mmol/L at 2 months and 26.61 ± 1.70 mmol/L at 5 months, respectively. As shown in Fig. 4, cardiac Nrf2 expression was slightly increased at 2 months (Fig. 4*A*) but significantly decreased at 5 months (Fig. 4*B*) after diabetes onset. The decreased cardiac Nrf2 expression at the late stage was accompanied with oxidative and nitrosative damage, shown by significantly increased cardiac 3-NT accumulation (Fig. 4*C*) and overactivation of ERK function (Fig. 4*D*), as observed in the in vitro study (Fig. 3). Importantly, all these pathogenic changes were not observed in the hearts of the MT-TG diabetic mice (Fig. 4*B–D*). These findings suggest that diabetic oxidative and nitrosative stress may induce ERK activation that downregulates Nrf2 expression.

Consistent with the finding from diabetic mice, immunochemical staining of human normal and diabetic hearts also showed that Nrf2 protein expression was dramatically suppressed in the diabetic failing hearts of both males (Fig. 5*A*) and females (Fig. 5*B*), as compared with nondiabetic hearts.

Pharmacological activation of Nrf2 attenuates oxidative stress–induced insulin resistance in adult cardiomyocytes in vitro and in the diabetic heart in vivo. We recently demonstrated that a novel synthetic triterpenoid derivative, Dh404, is a potent Nrf2 activator and suppresses oxidative stress in cardiomyocytes (20). We next determined whether Dh404 could mimic the inhibitory effect of Nrf2 overexpression on H_2O_2-induced insulin resistance in HL-1 cells. As expected, Dh404 strongly activated Nrf2 transcriptional activity and additively increased

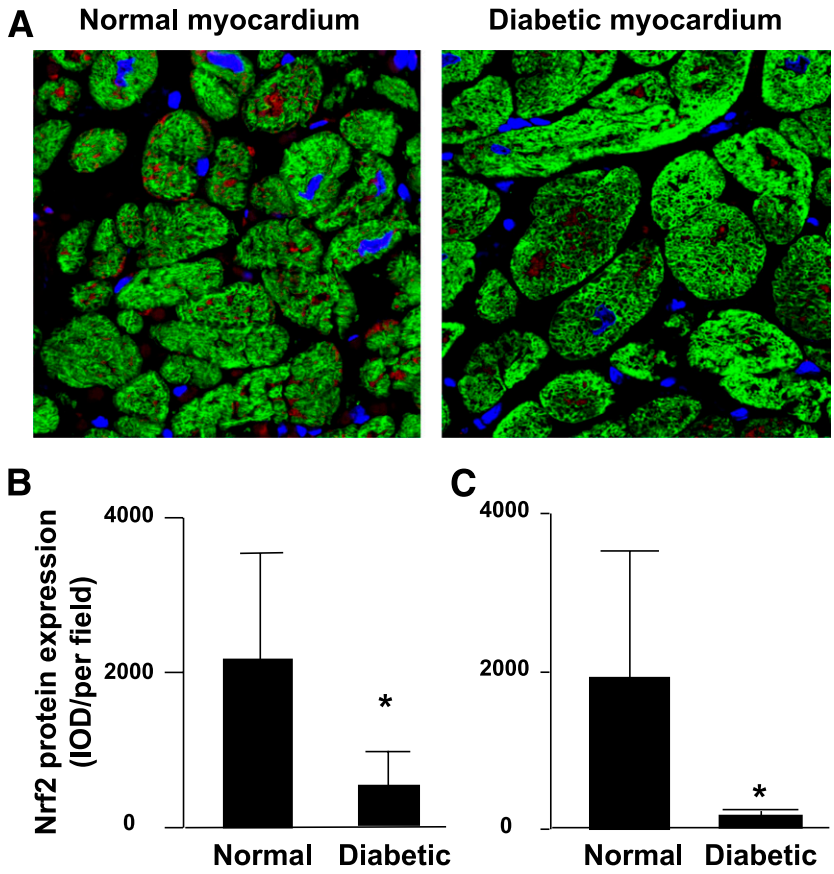

FIG. 5. Downregulation of Nrf2 expression in human diabetic hearts. *A*: Representatives of Nrf2 staining on left ventricular tissue sections of normal (males, *n* = 5; females, *n* = 5) and diabetic (males, *n* = 4; females, *n* = 2) human hearts. Red is Nrf2, and green is α-myosin heavy chain. Blue is nuclei. The semiquantification of Nrf2 protein levels by measuring mean integrated optical density (IOD) of eight randomly chosen fields of each tissue section for males (*B*) and females (*C*) are presented, respectively. Two sections of each heart specimen have been analyzed. **P* < 0.05 vs. normal group. (A high-quality digital representation of this figure is available in the online issue.)

the insulin-induced Nrf2 transcriptional activity (Fig. 6*A*). Dh404 treatment did not change insulin-induced ERK phosphorylation (Fig. 6*B*) and glucose uptake (Fig. 6*C*) but inhibited H_2O_2-induced ERK phosphorylation and partially reversed H_2O_2-inhibited basal glucose uptake or insulin-stimulated glucose uptake (Fig. 6*B* and *C*). These results further support the notion that Nrf2 is a critical regulator of insulin-mediated glucose uptake in cardiomyocytes in vitro.

To validate the in vitro finding, diabetic mice, induced by multiple-low doses of STZ, were treated with Dh404 at 10 mg/kg every other day for 2 weeks. Cardiac tissues were collected from these diabetic mice with and without Dh404 treatment and subject to Western blotting analysis for Nrf2 and 3-NT. Dh404 treatment did not change glucose levels of these mice (control 8.40 ± 1.20; diabetes: 25.40 ± 2.89; Dh404: 7.99 ± 0.56; diabetes/Dh404: 27.12 ± 1.22 mmol/L). Cardiac Nrf2 expression was increased in the hearts of either diabetic or Dh404-treated nondiabetic mice and synergistically increased in the hearts of Dh404-treated diabetic mice (Fig. 7*A*). Diabetes significantly induces 3-NT accumulation (Fig. 7*B*) and ERK1/2 phosphorylation (Fig. 7*C*), which were prevented by Dh404 treatment, suggesting that activation of Nrf2 expression by Dh404 in the heart plays an important role in the prevention of diabetes-induced cardiac oxidative and/or nitrosative damage and ERK1/2 activation. As expected, diabetes-induced oxidative damage and ERK1/2 activation is accompanied with downregulation of insulin signaling, shown by decreased phosphorylation of Akt (Fig. 7*D*) and GSK-3β (Fig. 7*E*). Activation of Nrf2 by Dh404 preserved the normal levels of Akt and GSK-3β phosphorylation in the heart of diabetic mice (Fig. 7*D* and *E*).

DISCUSSION

Oxidative stress is causative of insulin resistance in various cell types (2,6,7,18), which was confirmed by our present study using HL-1 adult cardiomyocytes treated with H_2O_2. Reportedly the stimulating effect of acute treatment with H_2O_2 on glucose uptake has been recognized for years in adipocytes (21). In fact, the cellular generation of ROS such as H_2O_2 by insulin has been considered as an integral part of the insulin signaling (21). These results emphasize the biological significance of ROS as signaling molecules, e.g., for the insulin-mediated glucose metabolism in cardiomyocytes; however, chronic accumulation of ROS causes oxidative stress, thereby contributing to the pathogenesis of various diseases such as cardiac dysfunction (9). Cardiac oxidative stress is often observed coincident with insulin resistance, and there is accumulating evidence that ROS and/or RNS mediates deleterious effects in the insulin-resistant heart although the mechanisms by which oxidative stress induces insulin resistance remain not well understood.

Increasing evidence indicates that ERK activation seems a causal factor for insulin resistance caused by oxidative stress in cardiomyocytes. For instance, through activation

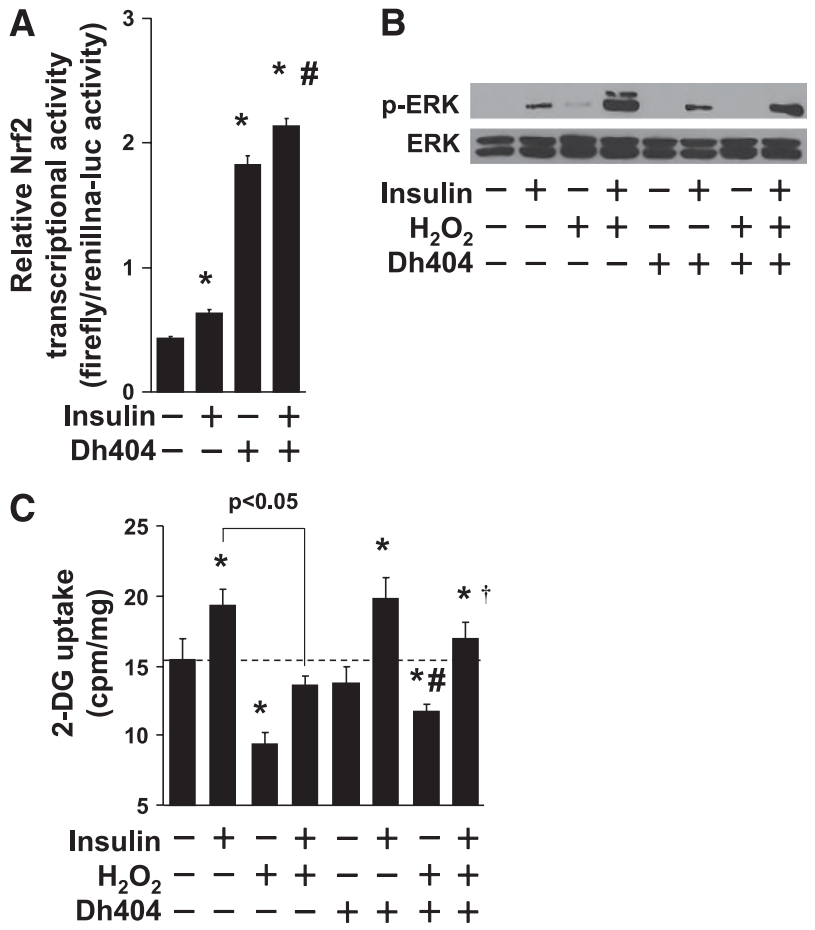

FIG. 6. A therapeutic effect of Dh404 on cardiomyocyte insulin resistance. *A*: Effect of Dh404 on insulin-induced Nrf2 transcriptional activity in HL-1 cells. Cells were transfected as in Fig. 3. The cells were stimulated with 100 nmol/L of insulin and 200 nmol/L of Dh404 as indicated for 12 h. Nrf2 transcriptional activity was measured as in Fig. 3. Effect of Dh404 on oxidative stress–induced ERK phosphorylation (*B*) and glucose uptake in HL-1 cells (*C*) is shown. Cells were pretreated with H_2O_2 (100 μmol/L) with or without Dh404 (200 nmol/L) in 0.1% FBS medium in the absence of norephineprine for 16 h and then stimulated with or without insulin (100 nmol/L for 10 min) as indicated. 2-DG uptake assay and Western blotting of phosphorylated and total ERK were performed as described in RESEARCH DESIGN AND METHODS. **P* < 0.05 vs. normal group; #*P* < 0.05 vs. insulin alone (*A*) or H_2O_2 alone (*C*); †*P* < 0.05 vs. H_2O_2 plus insulin (*C*).

of ERK1/2, high levels of glucose or angiotensin II induces insulin resistance in several conditions (19,22,23). A recent study that showed that deficiency in the ERK1 protects leptin-deficient mice from insulin resistance without affecting obesity further confirms the causative of ERK activation in the insulin resistance (24). In the current study, we demonstrated for the first time that chronic treatment of HL-1 cells with H_2O_2 increased ERK phosphorylation along with a significant inhibition of glucose uptake. More importantly we also demonstrated in the diabetic hearts that upregulation of ERK1/2 along with nitrosative damage is associated with downregulation of phosphorylation of both Akt and GSK-3β, two key components of insulin signaling cascade. In the cells with chronic H_2O_2 treatment, inhibition of ERK1/2 activation by U0126 was able to partially reverse glucose uptake and insulin resistance (Fig. 2*B*). Findings that NAC prevents H_2O_2-mediated activation of ERK1/2 in vitro (Fig. 1*B*) and MT prevents diabetes-induced activation of ERK1/2 in vivo (Fig. 4*D*) also suggest that the activation of ERK1/2 is associated with oxidative stress induced either by H_2O_2 in vitro or diabetes in vivo.

Nrf2 has been shown to negatively regulate oxidative stress in the heart (15). To support this notion, our recent study showed that high levels of glucose induced ROS production in both primary neonatal and adult cardiomyocytes from the heart of Nrf2 WT (C57BL/6 background) murine heart, whereas, in Nrf2 knockout cells, ROS was significantly higher under basal conditions and further enhanced under high glucose exposure conditions than that in WT cells (11). Concomitantly high glucose induced significantly higher levels of apoptosis at lower concentrations and in shorter time in Nrf2 knockout cells than in Nrf2 WT cells (11). To explore a pathological relevance of Nrf2 in diabetic hearts, we examined cardiac Nrf2 expression in STZ-induced diabetic mice. As shown in Figs. 4 and 7, cardiac Nrf2 expression was slightly increased in the heart of diabetic mice at 2 weeks (Fig. 7*A*) and 2 months (Fig. 4*A*) but significantly decreased in the hearts of diabetic mice at 5 months (Fig. 4*B*) after diabetes onset. The slight increase in cardiac Nrf2 expression at the early stages of diabetes was consistent with our previous study (11) in which Nrf2 was increased in the heart of diabetic mice with C57BL/6 background at 2 weeks after STZ-induced hyperglycemia. These results suggest that Nrf2 is adaptively trying to remain functional to overcome diabetic damage at the early stage of diabetes. At the late stage of diabetes, however, cardiac antioxidant function is further impaired, leading to a decrease in cardiac Nrf2 expression. Importantly, the decreased cardiac Nrf2

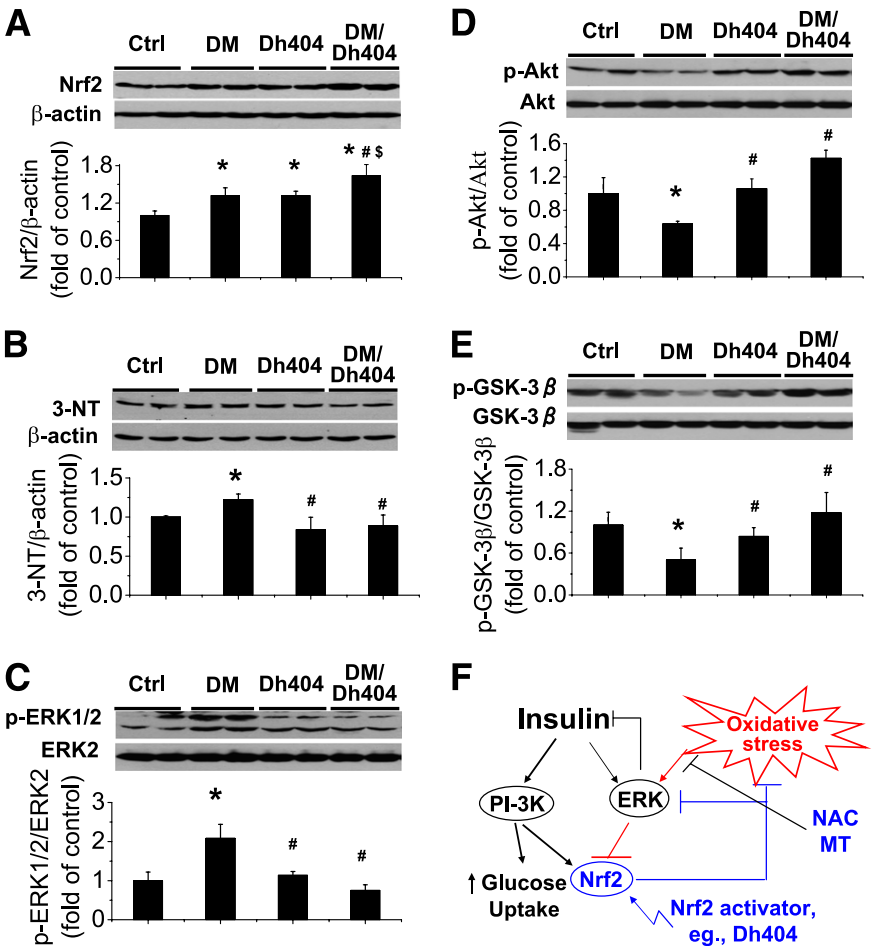

FIG. 7. Dh404 cardiac prevention of diabetic oxidative stress and inhibition of glucose metabolism. Diabetes was induced by multiple low doses of STZ as used in Fig. 4. Diabetic and age-matched nondiabetic mice were treated with Dh404 at 10 mg/kg body wt every other day from the onset of diabetes for 2 weeks. Cardiac Nrf2 expression (*A*); nitrosative damage, measured by 3-NT (*B*); ERK phosphorylation (*C*); Akt phosphorylation (*D*); and GSK-3β phosphorylation (*E*) were detected by Western blot, respectively ($n = 5$ for control [Ctrl], $n = 7$ for DM). β-Actin, total ERK, total Akt, or total GSK-3β was used as loading control. *$P < 0.05$ vs. control; #$P < 0.05$ vs. DM; \$$P < 0.05$ vs. Dh404. *F*: A working hypothesis of Nrf2-mediated regulation of insulin sensitivity in the cardiomyocytes. (A high-quality color representation of this figure is available in the online issue.)

expression in the diabetic hearts was accompanied with oxidative and nitrosative damage, shown by significantly increased 3-NT accumulation (Fig. 4*C*) and overactivation of ERK function (Fig. 4*D*) as observed in the in vitro study (Fig. 3). Peroxynitrite interacts with lipids, DNA, and proteins via direct nitration or via indirect, radical-mediated mechanisms. These reactions trigger cellular responses ranging from subtle modulations of cell signaling to overwhelming oxidative and nitrosative injury. In vivo, peroxynitrite generation represents a crucial pathogenic mechanism in conditions such as diabetic cardiomyopathy (12,25).

We have demonstrated that MT-TG mice are resistant to diabetes-induced oxidative and/or nitrosative damage and development of cardiomyopathy (13,26). Here we further showed that MT-TG mice are also significantly resistant to diabetes-induced decrease in cardiac Nrf2 expression (Fig. 4*B*) and increases in 3-NT accumulation (Fig. 4*C*) and ERK activation (Fig. 4*D*) at the late stage of diabetes (5 months after diabetes onset). Our finding is consistent with a recent study in which MT-TG mice are resistant to lipopolysaccharide (LPS)-induced cardiac oxidative stress, ERK1/2 activation, and cardiac dysfunction as compared with WT mice (27). Therefore, like NAC that protects cardiomyocytes from H_2O_2 downregulation of Nrf2 in the in vitro study (Fig. 3), MT as a potent antioxidant prevents diabetic oxidative and nitrosative stress, leading to a suppression of ERK activation and a preservation of cardiac Nrf2 function, as illustrated in Fig. 7*F*, to protect the heart from diabetes- and LPS-induced damage. We recently also showed that inactivation of GSK-3β by overexpression of cardiac *MT* gene results in an improvement of cardiac glucose metabolism in the diabetic hearts along with a significant suppression of cardiac nitrosative damage, inflammation, and remodeling (14).

In the current study, we demonstrated for the first time that Nrf2 plays a critical role in insulin-mediated glucose uptake, especially under an oxidative status. By Nrf2 transcription reporter assay we found that insulin activated Nrf2 (Fig. 3*A*), which could be enhanced by inhibition of ERK1/2 with U0126, whereas it is blocked by PI3K inhibition with LY294002 (Fig. 3*A*), indicating that insulin activates Nrf2 via PI3K-mediated signaling that is negatively regulated by ERK in HL-1 cells, as illustrated in Fig. 7*F*. Furthermore, forced activation of Nrf2 by adenoviral overexpression of Nrf2 significantly suppressed insulin-induced ERK activity in HL-1 cells with and without H_2O_2. This finding suggests that Nrf2 activation could prevent

oxidative stress–induced ERK activity in adult cardiomyocytes (Fig. 7*F*). Most importantly, the forced activation of Nrf2 further enhances insulin-mediated glucose uptake in non–H_2O_2-treated cells and also completely recovered insulin-mediated glucose uptake in H_2O_2-treated cells (Fig. 3*C*). The in vitro finding was further supported by in vivo results showing that diabetes inhibits cardiac Akt and GSK-3β phosphorylation, effects that were prevented by enhanced expression of Nrf2 with Dh404 (Fig. 7*D* and *E*). Therefore, these results not only establish a key role of Nrf2 in the maintaining of insulin sensitivity but also raise a possibility that Nrf2 is a crucial player in the regulation cardiac insulin signaling in the cardiomyocytes and the heart, as illustrated in Fig. 7*F*. These findings provide further support for other recent findings implying the link of Nrf2 deficiency to oxidative stress and insulin resistance in other cells and tissues (28,29). Therefore, diabetes-induced oxidative stress suppresses Nrf2 expression (Fig. 5) (30), and lack of Nrf2 expression in the diabetic hearts and kidney contributes to an insulin resistance and eventually cardiac dysfunction (11) or renal dysfunction (31,32).

In summary, our data provide several novel findings of redox signaling in the regulation of insulin sensitivity in the cardiac cells in vitro and in vivo, as illustrated by Fig. 7*F*. First, oxidative stress plays a causative role in the development of insulin resistance. Second, ERK is a negative regulator of glucose uptake and mediates oxidative stress–induced insulin resistance. Third, ERK-mediated suppression of Nrf2 activity is linked to the oxidative stress–induced insulin resistance. Fourth, Nrf2 expression is upregulated at early stage of diabetic heart but significantly downregulated at the late stage in diabetic hearts. Fifth, Nrf2 activation suppresses the oxidative stress–induced ERK activity and reverses the oxidative stress–induced insulin resistance. Finally, antioxidants such as NAC and MT can prevent oxidative stress–induced ERK activation and Nrf2 downregulation. These results suggest that Nrf2 plays a critical role in regulating insulin sensitivity in the heart, and targeting Nrf2 might provide a novel therapeutic approach for the treatment of cardiac insulin resistance and diabetic cardiomyopathy. Importantly a close structural analog of Dh404 that also potently induces Nrf2, bardoxolone methyl (CDDO-Me; RTA 402), is in a pivotal clinical study in patients with type 2 diabetes mellitus with advanced kidney disease to assess changes in renal function and glucose control (33,34).

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REFERENCES

1. Davidoff AJ, Davidson MB, Carmody MW, Davis ME, Ren J. Diabetic cardiomyocyte dysfunction and myocyte insulin resistance: role of glucose-induced PKC activity. Mol Cell Biochem 2004;262:155–163
2. Mellor KM, Ritchie RH, Delbridge LM. Reactive oxygen species and insulin-resistant cardiomyopathy. Clin Exp Pharmacol Physiol 2010;37:222–228
3. Doehner W, Rauchhaus M, Ponikowski P, et al. Impaired insulin sensitivity as an independent risk factor for mortality in patients with stable chronic heart failure. J Am Coll Cardiol 2005;46:1019–1026
4. Ingelsson E, Sundström J, Arnlöv J, Zethelius B, Lind L. Insulin resistance and risk of congestive heart failure. JAMA 2005;294:334–341
5. Björnholm M, Kawano Y, Lehtihet M, Zierath JR. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. Diabetes 1997;46:524–527
6. Ritchie RH. Evidence for a causal role of oxidative stress in the myocardial complications of insulin resistance. Heart Lung Circ 2009;18:11–18
7. Dong F, Fang CX, Yang X, Zhang X, Lopez FL, Ren J. Cardiac overexpression of catalase rescues cardiac contractile dysfunction induced by insulin resistance: role of oxidative stress, protein carbonyl formation and insulin sensitivity. Diabetologia 2006;49:1421–1433
8. Koca SS, Karaca I, Yavuzkir MF, et al. Insulin resistance is related with oxidative stress in systemic lupus erythematosus. Anadolu Kardiyol Derg 2009;9:23–28
9. Li J, Ichikawa T, Janicki JS, Cui T. Targeting the Nrf2 pathway against cardiovascular disease. Expert Opin Ther Targets 2009;13:785–794
10. Cheng X, Siow RC, Mann GE. Impaired redox signaling and antioxidant gene expression in endothelial cells in diabetes: a role for mitochondria and the nuclear factor-E2-related factor 2-kelch-like ECH-associated protein 1 defense pathway. Antioxid Redox Signal. 20 August 2010 [Epub ahead of print]
11. He X, Kan H, Cai L, Ma Q. Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. J Mol Cell Cardiol 2009;46:47–58
12. Cai L, Wang J, Li Y, et al. Inhibition of superoxide generation and associated nitrosative damage is involved in metallothionein prevention of diabetic cardiomyopathy. Diabetes 2005;54:1829–1837
13. Cai L, Wang Y, Zhou G, et al. Attenuation by metallothionein of early cardiac cell death via suppression of mitochondrial oxidative stress results in a prevention of diabetic cardiomyopathy. J Am Coll Cardiol 2006;48:1688–1697
14. Wang Y, Feng W, Xue W, et al. Inactivation of GSK-3beta by metallothionein prevents diabetes-related changes in cardiac energy metabolism, inflammation, nitrosative damage, and remodeling. Diabetes 2009;58:1391–1402
15. Li J, Ichikawa T, Villacorta L, et al. Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. Arterioscler Thromb Vasc Biol 2009;29:1843–1850
16. Claycomb WC, Lanson NA Jr, Stallworth BS, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci USA 1998;95:2979–2984
17. Ichikawa T, Li J, Nagarkatti P, et al. American ginseng preferentially suppresses STAT/iNOS signaling in activated macrophages. J Ethnopharmacol 2009;125:145–150
18. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006;440:944–948
19. Alfarano C, Sartiani L, Nediani C, et al. Functional coupling of angiotensin II type 1 receptor with insulin resistance of energy substrate uptakes in immortalized cardiomyocytes (HL-1 cells). Br J Pharmacol 2008;153:907–914
20. Ichikawa T, Li J, Meyer CJ, Janicki JS, Hannink M, Cui T. Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes. PLoS ONE 2009;4:e8391
21. Goldstein BJ, Mahadev K, Wu X, Zhu L, Motoshima H. Role of insulin-induced reactive oxygen species in the insulin signaling pathway. Antioxid Redox Signal 2005;7:1021–1031
22. Izawa Y, Yoshizumi M, Fujita Y, et al. ERK1/2 activation by angiotensin II inhibits insulin-induced glucose uptake in vascular smooth muscle cells. Exp Cell Res 2005;308:291–299
23. Nazari H, Takahashi A, Harada N, et al. Angiotensin II inhibits insulin-induced actin stress fiber formation and glucose uptake via ERK1/2. J Med Invest 2007;54:19–27
24. Jager J, Corcelle V, Gremeaux T, et al. Deficiency in the extracellular signal-regulated kinase 1 (ERK1) protects leptin-deficient mice from insulin resistance without affecting obesity. Diabetologia 2011;54:180–189

25. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424
26. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes 2002;51:1938–1948
27. Ceylan-Isik AF, Zhao P, Zhang B, Xiao X, Su G, Ren J. Cardiac overexpression of metallothionein rescues cardiac contractile dysfunction and endoplasmic reticulum stress but not autophagy in sepsis. J Mol Cell Cardiol 2010;48:367–378
28. Beyer TA, Xu W, Teupser D, et al. Impaired liver regeneration in Nrf2 knockout mice: role of ROS-mediated insulin/IGF-1 resistance. EMBO J 2008;27:212–223
29. Aleksunes LM, Reisman SA, Yeager RL, Goedken MJ, Klaassen CD. Nuclear factor erythroid 2-related factor 2 deletion impairs glucose tolerance and exacerbates hyperglycemia in type 1 diabetic mice. J Pharmacol Exp Ther 2010;333:140–151
30. Morrison CD, Pistell PJ, Ingram DK, et al. High fat diet increases hippocampal oxidative stress and cognitive impairment in aged mice: implications for decreased Nrf2 signaling. J Neurochem 2010;114:1581–1589
31. Yoh K, Hirayama A, Ishizaki K, et al. Hyperglycemia induces oxidative and nitrosative stress and increases renal functional impairment in Nrf2-deficient mice. Genes Cells 2008;13:1159–1170
32. Jiang T, Huang Z, Lin Y, Zhang Z, Fang D, Zhang DD. The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. Diabetes 2010;59:850–860
33. Thimmulappa RK, Fuchs RJ, Malhotra D, et al. Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. Antioxid Redox Signal 2007;9:1963–1970
34. Yates MS, Tauchi M, Katsuoka F, et al. Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. Mol Cancer Ther 2007;6:154–162