UC Irvine

UC Irvine Previously Published Works

Title

Pharmacological activation of Nrf2 pathway improves pancreatic islet isolation and transplantation.

Permalink

<https://escholarship.org/uc/item/21d1q4wr>

Journal

Cell transplantation, 24(11)

ISSN

0963-6897

Authors

Li, Shiri
Vaziri, Nosratola D
Masuda, Yuichi
et al.

Publication Date

2015

DOI

10.3727/096368915x686210

Peer reviewed

Pharmacological Activation of Nrf2 Pathway Improves Pancreatic Islet Isolation and Transplantation

Shiri Li,* Nosratola D. Vaziri,† Yuichi Masuda,* Mohammad Hajighasemi-Ossareh,* Lourdes Robles,* Aimee Le,* Kelly Vo,* Jefferson Y. Chan,‡ Clarence E. Foster,* Michael J. Stamos,* and Hirohito Ichii*

*Department of Surgery, University of California, Irvine, CA, USA

†Department of Medicine, University of California, Irvine, CA, USA

‡Department of Pathology and Laboratory Medicine, University of California, Irvine, CA, USA

Oxidative stress is a major cause of islet damage and loss during the islet isolation process. The Nrf2 pathway plays a critical role in protecting the cells against oxidative stress. The aim of this study was to investigate the effect of an Nrf2 activator (dh404) on islet isolation and transplantation in a rodent model. Islet isolation was conducted using Nrf2-deficient and wild-type mice and vehicle-treated and Nrf2 activator (dh404)-treated rats. Islet yield, viability, and Nrf2 pathway activity were determined. An *in vivo* islet potency test was done. Islet yield and viability in Nrf2-deficient mice was significantly lower compared to wild-type ($p < 0.05$) mice. Furthermore, administration of dh404 to normal Sprague–Dawley rats enhanced nuclear translocation of Nrf2 and elevated HO-1 expression in the pancreas. Islet yield and viability in dh404-treated rats was significantly higher compared to the vehicle-treated group ($p < 0.05$). The diabetes cure rate in nude mice with chemically induced diabetes was significantly greater in those transplanted with islets from the dh404-treated group (6/9) than vehicle-treated rats (2/9, $p < 0.05$). The Nrf2 pathway plays a significant role in protecting islets against stress caused by the isolation process. Pharmacological activation of the Nrf2 pathway significantly increased HO-1 expression, improved islet yield, viability, and function after transplantation.

Key words: Reactive oxygen species; Oxidative stress; Islet; Isolation; Transplantation; Nuclear erythroid 2-related factor 2 (Nrf2)

INTRODUCTION

Diabetes mellitus has become a critical disorder with major costs and complications worldwide. Successful islet transplantation is a promising therapy for select type 1 diabetes (T1DM) patients, due to substantial improvements of islet processing procedures and immunosuppressive medications (22,44,46). However, a major obstacle in this therapeutic approach is the insufficient yield of islets from deceased donor pancreata. An average islet isolation generally yields approximately 50% of the estimated over 1 million islets present in an adult human pancreas (26). Therefore, more than one donor pancreas is often needed for insulin independence after clinical islet transplantation (46,48).

It has been reported that oxidative stress is increased in patients with type 1 and type 2 diabetes. Studies have shown that biochemical markers of oxidative stress are higher in tissue samples and in the pancreas of patients with diabetes (45). When reactive oxygen species (ROS) are generated in excess for prolonged periods of time,

they cause chronic oxidative stress. Antioxidant enzymes contained within host tissue are the first line of defense against excessive levels of ROS. A vital consideration is the level of antioxidant host defense within the islet. It has been reported that the islet is among the least well-endowed tissue in terms of intrinsic antioxidant enzyme expression and activity (16,52). In addition, overexpression of antioxidant enzymes in insulinoma cells results in significantly increased resistance to cytokine-mediated toxicity (3). Transgenic mice that exhibit β -cell-targeted overexpression of an antioxidant enzyme were also shown to be resistant to autoimmune and streptozotocin-induced diabetes (19).

Pancreatic islets contain a very low level of antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase (30). Pancreatic islet isolation includes mechanical disruption, the chemical effects of collagenase, warm and cold ischemic injury, all of which increase ROS generation. These factors degrade islets and capillary membranes, which causes an additional release

of free radicals leading to apoptosis. Oxidative stress has been shown to play a major role in cell injury and β -cell dysfunction and death during islet isolation and transplantation (18,36,55).

Nuclear erythroid 2-related factor 2 (Nrf2) is a basic region leucine-zipper transcription factor, which binds to the antioxidant response element and regulates the expression of genes involved in the cellular antioxidant and anti-inflammatory defense. Nrf2 influences sensitivity to physiologic and pathologic processes affected by oxidative and electrophilic stress. The Nrf2-Kelch ECH associating protein 1 (Keap1) signaling pathway plays a significant role in protecting cells from various stresses including environmental agents/drug, inflammatory stresses, and chronic exposures to cigarette smoke and other carcinogens (8,12,13,27,28,31,32,38,51).

The aim of the present study was to investigate the effect of Nrf2 pathway activation on islets using a rodent islet isolation and transplant model. We hypothesize that activation of the Nrf2 pathway will contribute to the protection of islet cells against oxidative stress during islet isolation and following transplantation.

MATERIALS AND METHODS

Bardoxolone Methyl Analog (dh404)

RTA dh404 was provided by Reata Pharmaceuticals, Inc. (Irving, TX, USA). The chemical name for RTA dh404 is CDDO-9,11-dihydro-trifluoroethyl amide (CDDO-dhTFEA) (2,11,33,49,54,57).

Animals and Experiment Design

All animals were purchased from Charles River (Wilmington, MA, USA) or were received from collaborators and housed at our animal facility in accordance with the Institutional Animal Care and Use Committee of the University of California, Irvine (Irvine, CA, USA). Animals were maintained under 12-h light/dark cycles and allowed water and food ad libitum. Nrf2-deficient mouse (KO) on C58BL6; 129SV mix background have been described previously (5). To confirm the genotype from each animal, DNA was extracted from the tail and analyzed by PCR using the following primers: 3'-primer, 5'-GGAATGGAAAA-TAGCTCCTGCC-3'; 5'-primer, 5'-G CCTGAGAGCTGTAGGCC-3'; lacZ primer, 5'-GGG TTTTCCCAGTCACGAC-3'. Nrf2^{-/-} and Nrf2^{+/+} mice exhibited one band at 200 and 300 bp, respectively. Nine to 12-week-old mice (five mice in each group) were used in this study.

In a separate study, male Sprague-Dawley rats (250–300 g) were supplemented with oral Nrf2 activator, dh404. Rats were randomly divided into vehicle- and dh404-treated groups (10 rats in each group). Dh404 was dissolved in sesame oil (Sigma-Aldrich, St. Louis, MO, USA) and administered once a day by gavage at a dose of 0.6 mg/kg

body weight for 3 days prior to islet isolation. The control animals were fed vehicle (sesame oil) instead.

Streptozotocin (STZ)-Induced Diabetic Rat Model

Production of oxygen-free radicals and lipid peroxidation has been considered the main mechanisms by which STZ causes β -cell damage. To determine the protective effect on islets of dh404 against oxidative stress and optimize the dose of dh404, an STZ-induced diabetes rat model was used. Dh404 was orally given to rats via gavage at a dose range from 0.1 to 2 mg/kg (five rats in each group). Briefly, the male Sprague-Dawley rats were administered with dh404 at a dose range of 0.1–2 mg/kg body weight for 3 days prior to STZ treatment. STZ (Sigma-Aldrich) at a dose of 50 mg/kg body weight was dissolved in a freshly prepared citrate buffer (0.05 mol/L; pH 4.5; Sigma-Aldrich) and given to rats via an intraperitoneal injection.

Islet Isolation

Under isoflurane (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) anesthesia, midline abdominal incisions were performed, and the pancreases were exposed. The bile duct at the level of the duodenum was located and clamped. Animals were then sacrificed under deep sedation by cutting the abdominal aorta. A 23-gauge needle (rats) or 30 gauge (mice) attached to a 10-cc syringe was used to puncture and intubate the common bile duct. The pancreas was slowly distended by intraductal injection of pre-cooled Hank's balanced salt solution (HBSS; Mediatech, Herndon, VA, USA) containing 1.0 mg/ml collagenase type V (Sigma-Aldrich) at a dose of 1.5–2 ml for mice and 7–8 ml for rats. After distension, the whole pancreas was harvested and incubated in a 50-ml conical tube at 37°C for 18 min. Subsequently, the digestion was stopped by the addition of 20 ml of cold HBSS, and the conical tube was shaken vigorously. The suspension filtrated through a metallic filter net was washed with HBSS three times and centrifuged (150×g, 2 min, 4°C). The islets were purified using a Ficoll density gradient (Mediatech, Inc.). Islets were then collected and washed in HBSS. The crude number of islets in each diameter class was determined by counting after diphenylthionarbazone (DTZ; MP Biomedicals, Santa Ana, CA, USA) staining using an optical graticule. This number was then converted to the standard number of islet equivalents (IEQs; diameter standardizing to 150 μ m) (15). The purity of islet was also calculated during islet counting using DTZ staining. To minimize the bias, the islet number and purity were counted by double-blind evaluations.

Determination of Mouse Islet Cell Viability

Fluorescein diacetate (FDA) and propidium iodide (PI) staining were performed after mouse islet isolation to determine islet cell viability. Briefly, 50 islets were incubated with 20 μ l PI (1:10; Invitrogen Corp., Grand Island,

NY, USA) for 10 min kept from light at room temperature. After washing with phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA, USA) three times, 20 μ l FDA (1:10; Sigma-Aldrich) was added and counted under fluorescence microscope (Nikon Eclipse TS100; Nikon Instruments Inc., NY, USA). Dead cells were stained red, and viable cells were stained green. The percentage of viable cells in each islet was assessed, and the average was taken from 50 islets per mouse (4).

Glucose-Stimulated Insulin Secretion (GSIS) Test

To determine the *in vitro* potency of isolated rat islets, the insulin secretory response to glucose was measured using a modified method previously described (47). Briefly, 20–30 islets were randomly transferred to a cell culture insert (Millipore Ltd., Billerica, MA, USA) with Krebs buffer (115 mM NaCl, 5.0 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, pH 7.4), 25 mM HEPES, 0.1% BSA fraction V (Sigma-Aldrich) containing 2.8 mM glucose, and incubated at 37°C for 1 h as preincubation. Thereafter, they were suspended three times for 60 min at 37°C in Krebs buffer with the addition of various glucose concentrations (basal I: 2.8 mM, stimulation: 28 mM, basal II: 2.8 mM, respectively). The supernatants were collected and stored at –20°C. The insulin level was measured using a Rat Insulin Enzyme-Linked Immunosorbent Assay kit (Mercodia Inc., Winston Salem, NC, USA). The stimulation index (SI) was calculated as the insulin released into the stimulation medium divided by insulin released into the basal I medium.

In Vivo Islet Potency Test

Islet transplantation into diabetic nude mice was performed as an *in vivo* assessment of islet potency ($n=9$). Male nude mice (8 weeks old) were used as recipients of islet transplantation. Diabetic nude mice were made by intraperitoneal injection of STZ (Sigma-Aldrich) at 200 mg/kg body weight. The mice were considered diabetic if their nonfasting blood glucose levels exceeded 350 mg/dl for 3 consecutive days. Diabetic nude mice were anesthetized by isoflurane, and the left kidney was exposed through a lumbar incision. A breach was made in the kidney capsule, and a polyethylene catheter was introduced through the breach and advanced beneath the kidney capsule to generate a subcapsular space. To minimize the influence of purity on islet transplantation, purified islets were handpicked under the microscope to remove remaining acinar tissue to obtain islets with 95% purity for transplant. After handpicking, the islet samples were retaken and recounted, 200 IEQs were slowly injected through the PE50 tube (Fisher Scientific) into the subcapsular space. After removing the PE50 tube, the opening was cauterized, and the kidney was repositioned, followed by suturing of muscle and skin. Daily blood glucose levels were

obtained via tail vein prick using a contour glucometer (Bayer Healthcare LLC, Whippany, NJ, USA). At 4 weeks after transplantation, the nephrectomy with islet grafts was performed to confirm the graft function. Success of transplantation was defined as maintenance of normoglycemia (<200 mg/dl) for at least 3 consecutive days and return of hyperglycemia (>250 mg/dl) after nephrectomy.

Determination of Rat Islet Cell Viability

Cell viability was determined with previously reported methods (20,21). Briefly, 500–800 rat islets were incubated in 1 ml accutase solution (Sigma-Aldrich) at 37°C for 10 min. Accutase was deactivated with cold fetal bovine serum (Sigma-Aldrich). Washed cells were transferred into filtered FACS tubes (BD Falcon, Franklin Lakes, NJ, USA) to remove undigested tissue. Cells were resuspended in PBS and stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; Life Technologies, Grand Island, NY, USA) for 30 min at 37°C. This was followed by 7-amino-actinomycin D (7-AAD; Life Technologies) stain on ice immediately prior to the FACS analysis. TMRE stain indicates mitochondrial membrane potential, and 7-AAD is DNA-binding dye. The percentage of 7-AAD⁺ cells was recorded for dead cells, and further analysis for TMRE⁺ (viable cells) and TMRE[–] (apoptotic cells) was performed after their exclusion (gating out). Fifty thousand cells from each sample were assessed by flow cytometry (Accuri C6 cytometers; BD Biosciences, Ann Arbor, MI, USA). Software (Accuri C6 software; BD Biosciences, San Jose, CA, USA) was used for analysis (20).

Western Blot Analysis

Total protein from rat pancreases (both vehicle- and dh404-treated groups) were obtained with CellLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich) using the manufacturer's instructions. In brief, pancreatic tissue was homogenized and lysed on ice with lysis buffer including DTT and protease inhibitors for 20 min. The pancreatic lysate was centrifuged at 11,000 $\times g$ for 11 min. The cytoplasmic protein concentration was measured using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The primary antibodies used were rabbit antibodies against Heme oxygenase-1 (HO-1; 1:1,000 dilution; Abcam Inc., Cambridge, MA, USA) and GAPDH (1:5,000 dilution; Sigma-Aldrich). Aliquots containing 15 μ g proteins were loaded on NuPAGE 4–12% Bis-Tris gel (Life Technologies) and then were transferred to a PVDF membrane (Pall Life Science, Ann Arbor, MI, USA). The membrane was blocked in TBS-T (Thermo Scientific, West Palm Beach, FL, USA) containing 5% blocking-grade nonfat dry milk (Bio-Rad Laboratories), and incubated with primary antibodies overnight at 4°C. After washing, the membrane was further incubated with HRP-conjugated goat anti-rabbit secondary antibody

(1:3,000 dilution; Cell Signaling, Danvers, MA, USA) at room temperature for 2 h. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA). Densitometric measurements were done with Image Quant (GE Healthcare Life Sciences, Pittsburgh, PA, USA). GAPDH bands, as a housekeeping protein, were used as an internal control.

Immunofluorescence and Confocal Microscopy

Rat islets were fixed in 10% formalin (Fisher Scientific) and embedded in optical cutting temperature compound (Sakura Finetek Inc., Torrance, CA, USA) and frozen; then 10- μ m-thick sections were cut, rinsed in PBS and 5% BSA, and incubated with Universal Blocker Reagent (Biogenex, San Ramon, CA, USA) for 30 min in a humidified chamber at room temperature. Thereafter, sections were incubated with primary antibodies, rabbit anti-Nrf2 (1:100 dilution; Abcam Inc.) overnight at 4°C in a humidified chamber. After PBS wash, Alexa Fluor 488 antibody (1:100 dilution; Molecular Probes, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen Corp.) were applied to the slides and incubated at room temperature for 2 h in the dark. The slides were analyzed under a confocal laser scanning microscopy (Zeiss LSM700; Carl Zeiss, Jena, Germany), and the images were analyzed by ZEN 2011 (Carl Zeiss, Jena, Germany).

To analyze the percentage of β -cells in islet, the fresh isolated rat islets were dissociated into single cells, and

they were fixed on glass slides with 2.5% paraformaldehyde (Sigma-Aldrich). Cells were incubated with Universal Blocker Reagent (Biogenex) for 30 min, to reduce nonspecific binding. Thereafter, cells were incubated overnight at 4°C with mouse monoclonal antibody to insulin (1:200 dilution; Abcam). After washing, cells were incubated with goat anti-mouse IgG (Alexa Fluor 488 goat anti-mouse IgG, 1:200 dilution; Life Technologies). Omission of the primary antibody served as negative control. DAPI was applied to stain cell nuclei. The cellular composition was determined by manually counting the stained cells using a fluorescent microscope. β -Cell content was determined by taking the number of positively stained β -cells and dividing by the total number of stained cells (20,21)

Statistical Analysis

Results were expressed as mean $\pm$ standard deviation (SD), and the statistical differences between groups were determined by an unpaired *t*-test. The percentage of cured diabetic nude mice between two groups was determined by Gehan–Breslow–Wilcoxon test. Values of $p < 0.05$ were considered significant.

RESULTS

Islet Yield and Viability of Nrf2-Deficient Mice

To investigate the effect of Nrf2 deficiency on the pancreas, islet isolation was performed in Nrf2-deficient mice. Interestingly, the islet yield in the Nrf2-deficient

Figure 1. Islet yield and viability of Nrf2-deficient mice. (A) Islet yields. The islet yield in the wild-type group was significantly higher than that in Nrf2 KO group ($*p < 0.05$). (B) Islet number. There was no significant difference between the groups. (C) Islet viability. The percentage of viable cells in the wild-type group was significantly higher than that in Nrf2 KO group ($*p < 0.05$). Data are expressed as mean $\pm$ SD.

group was significantly lower when compared to the wild-type group (15.6 ± 4.9 IEQs/g body weight vs. 24.2 ± 1.91 IEQs/g body weight), although islet number was comparable (Fig. 1A, B). The islet viability assessed by FDA/PI in Nrf2-deficient islets ($84.5 \pm 0.6\%$) was also significantly lower when compared to wild-type islets ($93.0 \pm 3.8\%$) (Fig. 1C).

Adjustment of dh404 Dose Using STZ-Induced Diabetic Rat Model

An STZ-induced diabetic rat model was used to determine an optimal dose of dh404 in vivo for islet protection against oxidative stress during islet isolation. Dh404 was orally given to rats via gavage at a dose range from 0.1 to 2 mg/kg. Figure 2 shows blood glucose level after STZ injection in the rats treated at 0.1, 0.6, and 2 mg/kg body weight. The results revealed that the dose of dh404 between 0.5 and 1.5 mg/kg was beneficial for the β -cell protection from STZ. However, higher doses (2 mg/kg) of dh404 did not show protective effects in our STZ-induced diabetic rat model.

Pharmacological Activation of Nrf2 Pathway by dh404 in Rodent Pancreases

Nrf2 translocation to the nucleus was evaluated to confirm the upregulation of Nrf2 after dh404 oral administration. The pancreas of dh404-supplemented and vehicle-treated rats were stained with Nrf2 antibody and DAPI and then assessed by confocal microscope. Compared to the control group, Nrf2 translocation to the nucleus of pancreatic tissue in the dh404-treated rats was clearly demonstrated (Fig. 3A). In addition, the expression of HO-1, one of the target antioxidant enzymes of the Nrf2 pathway, was examined in the pancreases of the rats by Western blot. Dh404 treatment produced a 10-fold increase in HO-1 expression compared to the vehicle-treated controls (Fig. 3B).

Nrf2 Pathway Activation in dh404-Treated Islets

To investigate the effect of pharmacological activation of the Nrf2 pathway on islet isolation, dh404 (0.6 mg/kg once/day) was administrated orally to rats for 3 days prior to isolation. The isolated islets were stained

Figure 2. Adjustment of dh404 dose using streptozotocin (STZ)-induced diabetes model. STZ-induced diabetes model was used to determine an optimal dose of dh404. The 0.5–1.5 mg/kg dose demonstrated beneficial effects on STZ-induced diabetes. Higher doses (more than 2 mg/kg) of dh404 did not show protective effects in this model.

Figure 3. (A) Nrf2 translocation in pancreas. The pancreases of dh404-supplemented rats were stained with Nrf2 antibody and DAPI and then assessed by confocal microscope. Nrf2 translocation (merged blue and green) to the nucleus in the dh404-treated rats was clearly demonstrated (blue = nuclei, green = Nrf2, arrows showing Nrf2 translocation). (B). HO-1 expression in dh404-supplemented rat pancreas. Dh404-treated pancreas produces a further by 10-fold increase in HO-1 expression.

with Nrf2 antibody and DAPI and then assessed by confocal microscope. Nrf2 translocation to the nucleus of islets in the dh404-treated group was clearly shown (Fig. 4).

Administration of dh404 Improves Islet Yield and Purity

The islet yield and islet number in the dh404-treated group (1522.6 ± 434.8 IEQs) was significantly higher than those in the vehicle-treated group (1192.4 ± 233.4 IEQs, $p < 0.05$) (Fig. 5 A, B). Moreover, the purity of islets in the dh404-treated group ($91.5 \pm 3.72\%$) was significantly higher compared to the vehicle-treated animals ($85 \pm 3.78\%$, $p < 0.01$) (Fig. 5C). Stimulation index in the dh404-treated group (1.77 ± 0.34) was comparable to the vehicle-treated group (1.6 ± 0.27) (Fig. 5D).

Islet Viability and Cellular Composition

Single islet cell suspensions were incubated with 7-AAD and TMRE after dissociating freshly isolated islets. Apoptosis and necrosis in islet cells were analyzed by flow cytometer. The percentage of apoptotic cells (TMRE⁻) in the dh404-treated group ($18.3 \pm 2.1\%$)

was significantly lower than in the vehicle-treated group ($23.4 \pm 2.1\%$, $p < 0.05$) (Fig. 6A). However, there was no significant difference in necrotic cells between the experimental groups ($7.44 \pm 3.24\%$ vs. $5.95 \pm 2.19\%$). Moreover, the percentage of β -cells in islets was analyzed to determine the influence of Nrf2 pathway activation on islet survival. As shown in Figure 6B, the percentage of β -cells in the dh404-treated group was significantly higher when compared with the vehicle-treated groups ($70.2 \pm 4.7\%$ vs. $65.9 \pm 4.0\%$, $p < 0.05$).

In Vivo Islet Potency Test

Marginal mass of rat islets (200 IEQs/mouse) in each group were transplanted into the kidney capsule of nude mice with STZ-induced diabetes to determine the effect of dh404 on islet function in vivo (Fig. 7). The reversal rate of diabetes in the dh404-treated group was significantly higher than the vehicle-treated group (6/9 vs. 2/9, $p = 0.034$). The nephrectomy of the islet-grafted kidney was performed at 4 weeks after transplantation to confirm the graft function.

Figure 4. Nrf2 translocation in isolated islets. The isolated islets from dh404-supplemented rats were stained with Nrf2 antibody and DAPI and then assessed by confocal microscope. Nrf2 translocation (merged blue and green) to the nucleus of islets in the dh404-treated group was clearly demonstrated (blue=nuclei, green=Nrf2, arrows showing Nrf2 translocation).

DISCUSSION

Obtaining a consistently high islet yield and viability is a prerequisite to succeed with islet transplantation. In clinical settings, only 20–50% of potential islet mass in a human pancreas are generally recovered even with the best islet isolation procedure and by well-experienced operators (44). Oxidative stress is a major factor potentially causing islet damage and loss during the islet isolation process (42,43). ROS have a great potential to directly or indirectly disturb the cellular integrity and physiological function of biomolecules like lipids, proteins, and DNA and have been implicated in various diseases, including atherosclerosis, pulmonary fibrosis, cancer, and adult respiratory distress syndrome (1,17). Pancreatic islets have low activity of free radical detoxifying enzymes when compared to other tissue (16,30,37). Several studies have shown that administration of antioxidants like curcumin, quercetin, and probucol could protect islet cells from damage by free radicals (9,14,35). In addition, as one of the strategies against oxidative stress, overexpression of genes responsible for antioxidant enzymes in islets have exhibited better protection against damage caused by free radicals (1). Nrf2 induction prevents reactive species damage in pancreatic β -cells, and the Keap1 Nrf2 system is the crucial defense pathway for

the physiological and pathological protection of pancreatic β -cells (56). In the current study, we demonstrated the protective effect of Nrf2 activator dh404 on islet cells during the islet isolation procedure. Islet yield and viability were found to be significantly reduced in Nrf2-deficient pancreas. Furthermore, pharmacological Nrf2 activation by dh404 resulted in significantly better islet yields, viability, and β -cell survival, leading to higher reversal rate of diabetes in islet transplantation into diabetic immunodeficient mice. Nrf2 is a transcription factor important in the protection against oxidative stress through the antioxidant response element (ARE)-mediated transcriptional activation of several phase II detoxifying and antioxidant enzymes. It is generally thought that dh404 allows Nrf2 to translocate to the nucleus where it upregulates expression of antioxidant and cytoprotective genes (11,49,54). Nrf2 has a significant protective role against oxidative injury, through transcriptional activation of genes encoding antioxidant enzymes and related proteins (6,7). Our results clearly indicated that dh404 treatment promotes translocation of Nrf2 from the cytoplasm into the nucleus in isolated islet cells (see Fig. 4A). Moreover, it upregulated HO-1 expression in pancreatic tissue. HO-1 has been implicated as a promising therapeutic target in various diseases

Figure 5. Islet isolation in dh404-treated rat. (A) Islet yield. The islet yield in the vehicle-treated group was significantly lower compared to the dh404-supplemented group (* $p < 0.05$). (B) Islet number. The islet number in the vehicle-treated group was significantly lower compared to the dh404-supplemented group (* $p < 0.05$). (C) Islet purity. Islet purity in the dh404-supplemented group was significantly higher. (D) Glucose-stimulated insulin release. There was no significant difference between groups. Data are expressed as mean $\pm$ SD.

Figure 6. (A) Rat islet viability. Islets in dh404-supplemented rats showed less apoptotic cells compared to the vehicle-treated group (* $p < 0.05$). There was no difference in dead cells between these two groups. (B) Cellular composition in islet. Islets in dh404-supplemented rats showed a significantly higher percentage of β -cells in islet compared to the vehicle-treated group (* $p < 0.05$). Data are expressed as mean $\pm$ SD.

Figure 7. In vivo islet potency tests. Islets (200 IEQs/mouse) in each group were transplanted into kidney capsule of STZ-induced diabetic nude mice to determine the effect of dh404 on islet function in vivo. The reversal rate of diabetes in the dh404-treated group was significantly higher than the vehicle-treated group (6/9 vs. 2/9, $p=0.034$). The nephrectomy of the islet-grafted kidney was performed at 4 weeks after transplantation to confirm the graft function.

(10,50). A study in HO-1-deficient mice has shown HO-1 to play a critical role in the anti-inflammatory process (23). Furthermore, it has been reported that HO-1 upregulation led to protective effects on β -cells against various apoptotic stimuli including cytokines and Fas (41,53).

We performed GSIS with isolated islets to evaluate the effect of dh404 on islet function. The dh404-treated group showed no significant improvement. For clinical application, in vivo function is a most important evaluation after islet transplantation. Transplanted islets can be damaged by early inflammation at the site of implantation, which leads to ROS inducing either functional impairment or cell death (25,40). Our study demonstrated that HO-1 upregulation could improve graft function in vivo as well as another study (39). Further, the HO-1 system serves as a novel therapeutic concept in organ transplantation as the multifaceted targets of HO-1-mediated cytoprotection may simultaneously benefit both local graft function and host systemic immune response (24).

Compared to the liver, islets contain only 1% catalase, 2% GPX1, and 29% SOD1 activities. Thus, β -cells are considered to be low in antioxidant defense and are therefore susceptible to oxidative stress (29). In addition, Yogishita et al. recently reported that β -cell-specific Nrf2 conditional knockout mice strongly aggravated β -cell damage and that Nrf2 induction prevented reactive species-induced damage in β -cells (56). Therefore, the pharmacological activation of the Nrf2 pathway by dh404 significantly increased not only islet yield but also β -cell content in isolated islets, since it may be more effective in β -cells when compared to other endocrine cells.

β -Cells are the primary target of diabetogenic agents, such as STZ (34). To determine if dh404 treatment would

have an effect on β -cell mass after isolation, we analyzed β -cell content in isolated islets. A remarkable finding in our current study was that β -cell content was significantly increased in the dh404-supplemented group after isolation. In a human islet isolation study, authors showed β -cell mass to be directly related to the in vivo transplantation success rate (20). Our data showed that dh404 treatment had not only improved islet yield but also increased β -cell content in isolated islets after the isolation procedure.

A low dose of dh404 was administrated into rats for islet isolation in our current study. A recent study showed that dh404, unless administered acutely at low doses, is not well tolerated in rodents (58). The adjustment of the dose of dh404 in various targets may be crucial for clinical application of dh404. dh404 is not water soluble, but it can be dissolved in DMSO, which allows us to add dh404 into various media used for pancreas preservation, islet isolation, and culture. The effect of in vitro treatment of dh404 on islet isolation using pancreases from large mammals such as human, monkey, and pig will be investigated as the next step toward its potential utility in a clinical setting.

In summary, our data demonstrated that pretreatment with low-dose dh404 improved islet yield, viability, and β -cell content by promoting Nrf2 nuclear translocation and upregulating HO-1 in islet cells during the islet isolation procedure. Targeting Nrf2 activation may prove to be a therapeutic strategy for improved islet isolation and transplantation outcomes.

ACKNOWLEDGMENTS: This study was in part supported by grants from NIH-NCRR UL1 TR000153, KL2 TR000147; the Juvenile Diabetes Research Foundation International 17-2011-609. The authors declare no conflicts of interest.

REFERENCES

- Acharya, J. D.; Ghaskadbi, S. S. Islets and their antioxidant defense. *Islets* 2(4):225–235; 2010.
- Aminzadeh, M. A.; Reisman, S. A.; Vaziri, N. D.; Khazaeli, M.; Yuan, J.; Meyer, C. J. The synthetic triterpenoid RTA dh404 (CDDO-dhTFEA) restores Nrf2 activity and attenuates oxidative stress, inflammation, and fibrosis in rats with chronic kidney disease. *Xenobiotica* 44(6):570–578; 2014.
- Azevedo-Martins, A. K.; Lortz, S.; Lenzen, S.; Curi, R.; Eizirik, D. L.; Tiedge, M. Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor-kappaB activation in insulin-producing cells. *Diabetes* 52(1):93–101; 2003.
- Barnett, M. J.; McGhee-Wilson, D.; Shapiro, A. M.; Lakey, J. R. Variation in human islet viability based on different membrane integrity stains. *Cell Transplant.* 13(5):481–488; 2004.
- Chan, K.; Lu, R.; Chang, J. C.; Kan, Y. W. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. USA* 93(24):13943–13948; 1996.
- Cho, H. Y.; Jedlicka, A. E.; Reddy, S. P.; Kensler, T. W.; Yamamoto, M.; Zhang, L. Y.; Kleeberger, S. R. Role of NRF2 in protection against hyperoxic lung injury in mice. *Am. J. Respir. Cell Mol. Biol.* 26(2):175–182; 2002.
- Cho, H. Y.; Reddy, S. P.; Debiase, A.; Yamamoto, M.; Kleeberger, S. R. Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free Radic. Biol. Med.* 38(3):325–343; 2005.
- Copple, I. M.; Goldring, C. E.; Kitteringham, N. R.; Park, B. K. The Nrf2-Keap1 defence pathway: Role in protection against drug-induced toxicity. *Toxicology* 246(1):24–33; 2008.
- Coskun, O.; Kanter, M.; Korkmaz, A.; Oter, S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. *Pharmacol. Res.* 51(2):117–123; 2005.
- Deshane, J.; Wright, M.; Agarwal, A. Heme oxygenase-1 expression in disease states. *Acta Biochim. Pol.* 52(2):273–284; 2005.
- Dinkova-Kostova, A. T.; Liby, K. T.; Stephenson, K. K.; Holtzclaw, W. D.; Gao, X.; Suh, N.; Williams, C.; Risingsong, R.; Honda, T.; Gribble, G. W.; Sporn, M. B.; Talalay, P. Extremely potent triterpenoid inducers of the phase 2 response: Correlations of protection against oxidant and inflammatory stress. *Proc. Natl. Acad. Sci. USA* 102(12):4584–4589; 2005.
- Giudice, A.; Arra, C.; Turco, M. C. Review of molecular mechanisms involved in the activation of the Nrf2-ARE signaling pathway by chemopreventive agents. *Methods Mol. Biol.* 647:37–74; 2010.
- Giudice, A.; Montella, M. Activation of the Nrf2-ARE signaling pathway: A promising strategy in cancer prevention. *Bioessays* 28(2):169–181; 2006.
- Gorogawa, S.; Kajimoto, Y.; Umayahara, Y.; Kaneto, H.; Watada, H.; Kuroda, A.; Kawamori, D.; Yasuda, T.; Matsuhisa, M.; Yamasaki, Y.; Hori, M. Probulcol preserves pancreatic beta-cell function through reduction of oxidative stress in type 2 diabetes. *Diabetes Res. Clin. Pract.* 57(1):1–10; 2002.
- Gotoh, M.; Maki, T.; Kiyozumi, T.; Satomi, S.; Monaco, A. P. An improved method for isolation of mouse pancreatic islets. *Transplantation* 40(4):437–438; 1985.
- Grankvist, K.; Marklund, S. L.; Taljedal, I. B. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem. J.* 199(2):393–398; 1981.
- Halliwell, B.; Gutteridge, J. M.; Cross, C. E. Free radicals, antioxidants, and human disease: Where are we now? *J. Lab. Clin. Med.* 119(6):598–620; 1992.
- Hennige, A. M.; Lember, N.; Wahl, M. A.; Ammon, H. P. Oxidative stress increases potassium efflux from pancreatic islets by depletion of intracellular calcium stores. *Free Radic. Res.* 33(5):507–516; 2000.
- Hotta, M.; Tashiro, F.; Ikegami, H.; Niwa, H.; Ogihara, T.; Yodoi, J.; Miyazaki, J. Pancreatic beta cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. *J. Exp. Med.* 188(8):1445–1451; 1998.
- Ichii, H.; Inverardi, L.; Pileggi, A.; Molano, R. D.; Cabrera, O.; Caicedo, A.; Messinger, S.; Kuroda, Y.; Berggren, P. O.; Ricordi, C. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. *Am. J. Transplant.* 5(7):1635–1645; 2005.
- Ichii, H.; Miki, A.; Yamamoto, T.; Molano, R. D.; Barker, S.; Mita, A.; Rodriguez-Diaz, R.; Klein, D.; Pastori, R.; Alejandro, R.; Inverardi, L.; Pileggi, A.; Ricordi, C. Characterization of pancreatic ductal cells in human islet preparations. *Lab. Invest.* 88(11):1167–1177; 2008.
- Ichii, H.; Ricordi, C. Current status of islet cell transplantation. *J. Hepatobiliary Pancreat. Surg.* 16(2):101–112; 2009.
- Kapturczak, M. H.; Wasserfall, C.; Brusko, T.; Campbell-Thompson, M.; Ellis, T. M.; Atkinson, M. A.; Agarwal, A. Heme oxygenase-1 modulates early inflammatory responses: Evidence from the heme oxygenase-1-deficient mouse. *Am. J. Pathol.* 165(3):1045–1053; 2004.
- Katori, M.; Busuttil, R. W.; Kupiec-Weglinski, J. W. Heme oxygenase-1 system in organ transplantation. *Transplantation* 74(7):905–912; 2002.
- Kaufman, D. B.; Gores, P. F.; Field, M. J.; Farney, A. C.; Gruber, S. A.; Stephanian, E.; Sutherland, D. E. Effect of 15-deoxyspergualin on immediate function and long-term survival of transplanted islets in murine recipients of a marginal islet mass. *Diabetes* 43(6):778–783; 1994.
- Kin, T. Islet isolation for clinical transplantation. *Adv. Exp. Med. Biol.* 654:683–710; 2010.
- Kobayashi, M.; Yamamoto, M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox. Signal* 7(3–4):385–394; 2005.
- Kundu, J. K.; Surh, Y. J. Nrf2-Keap1 signaling as a potential target for chemoprevention of inflammation-associated carcinogenesis. *Pharm. Res.* 27(6):999–1013; 2010.
- Lei, X. G.; Vatamaniuk, M. Z. Two tales of antioxidant enzymes on beta cells and diabetes. *Antioxid. Redox. Signal* 14(3):489–503; 2011.
- Lenzen, S.; Drinkgern, J.; Tiedge, M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic. Biol. Med.* 20(3):463–466; 1996.
- Li, W.; Kong, A. N. Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol. Carcinog.* 48(2):91–104; 2009.
- Li, W.; Wu, W.; Song, H.; Wang, F.; Li, H.; Chen, L.; Lai, Y.; Janicki, J. S.; Ward, K. W.; Meyer, C. J.; Wang, X.; Tang, D.; Cui, T. Targeting Nrf2 by dihydro-CDDO-trifluoroethyl amide enhances autophagic clearance and viability of beta-cells in a setting of oxidative stress. *FEBS Lett.* 588(12):2115–2124; 2014.

33. Liby, K. T.; Yore, M. M.; Sporn, M. B. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. *Nat. Rev. Cancer* 7(5):357–369; 2007.
34. Like, A. A.; Rossini, A. A. Streptozotocin-induced pancreatic insulinitis: New model of diabetes mellitus. *Science* 193(4251):415–417; 1976.
35. Meghana, K.; Sanjeev, G.; Ramesh, B. Curcumin prevents streptozotocin-induced islet damage by scavenging free radicals: A prophylactic and protective role. *Eur. J. Pharmacol.* 577(1–3):183–191; 2007.
36. Miwa, I.; Ichimura, N.; Sugiura, M.; Hamada, Y.; Taniguchi, S. Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology* 141(8):2767–2772; 2000.
37. Modak, M. A.; Datar, S. P.; Bhonde, R. R.; Ghaskadbi, S. S. Differential susceptibility of chick and mouse islets to streptozotocin and its co-relation with islet antioxidant status. *J. Comp. Physiol. B* 177(2):247–257; 2007.
38. Pi, J.; Zhang, Q.; Fu, J.; Woods, C. G.; Hou, Y.; Corkey, B. E.; Collins, S.; Andersen, M. E. ROS signaling, oxidative stress and Nrf2 in pancreatic beta-cell function. *Toxicol. Appl. Pharmacol.* 244(1):77–83; 2010.
39. Pileggi, A.; Molano, R. D.; Berney, T.; Cattani, P.; Vizzardelli, C.; Oliver, R.; Fraker, C.; Ricordi, C.; Pastori, R. L.; Bach, F. H.; Inverardi, L. Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. *Diabetes* 50(9):1983–1991; 2001.
40. Rabinovitch, A.; Suarez-Pinzon, W. L.; Strynadka, K.; Lakey, J. R.; Rajotte, R. V. Human pancreatic islet beta-cell destruction by cytokines involves oxygen free radicals and aldehyde production. *J. Clin. Endocrinol. Metab.* 81(9):3197–3202; 1996.
41. Ribeiro, M. M.; Klein, D.; Pileggi, A.; Molano, R. D.; Fraker, C.; Ricordi, C.; Inverardi, L.; Pastori, R. L. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. *Biochem. Biophys. Res. Commun.* 305(4):876–881; 2003.
42. Robertson, R.; Zhou, H.; Zhang, T.; Harmon, J. S. Chronic oxidative stress as a mechanism for glucose toxicity of the beta cell in type 2 diabetes. *Cell Biochem. Biophys.* 48(2–3):139–146; 2007.
43. Robertson, R. P. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J. Biol. Chem.* 279(41):42351–42354; 2004.
44. Robertson, R. P. Islet transplantation as a treatment for diabetes - A work in progress. *N. Engl. J. Med.* 350(7):694–705; 2004.
45. Robertson, R. P. Oxidative stress and impaired insulin secretion in type 2 diabetes. *Curr. Opin. Pharmacol.* 6(6):615–619; 2006.
46. Ryan, E. A.; Lakey, J. R.; Paty, B. W.; Imes, S.; Korbutt, G. S.; Kneteman, N. M.; Bigam, D.; Rajotte, R. V.; Shapiro, A. M. Successful islet transplantation: Continued insulin reserve provides long-term glycemic control. *Diabetes* 51(7):2148–2157; 2002.
47. Sawada, T.; Matsumoto, I.; Nakano, M.; Kirchhof, N.; Sutherland, D. E.; Hering, B. J. Improved islet yield and function with ductal injection of University of Wisconsin solution before pancreas preservation. *Transplantation* 75(12):1965–1969; 2003.
48. Shapiro, A. M.; Lakey, J. R.; Ryan, E. A.; Korbutt, G. S.; Toth, E.; Warnock, G. L.; Kneteman, N. M.; Rajotte, R. V. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N. Engl. J. Med.* 343(4):230–238; 2000.
49. Sporn, M. B.; Liby, K. T.; Yore, M. M.; Fu, L.; Lopchuk, J. M.; Gribble, G. W. New synthetic triterpenoids: Potent agents for prevention and treatment of tissue injury caused by inflammatory and oxidative stress. *J. Nat. Prod.* 74(3):537–545; 2011.
50. Stocker, R.; Perrella, M. A. Heme oxygenase-1: A novel drug target for atherosclerotic diseases? *Circulation* 114(20):2178–2189; 2006.
51. Surh, Y. J.; Kundu, J. K.; Na, H. K. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med.* 74(13):1526–1539; 2008.
52. Tiedge, M.; Lortz, S.; Drinkgern, J.; Lenzen, S. Relation between antioxidant enzyme gene expression and antioxidant defense status of insulin-producing cells. *Diabetes* 46(11):1733–1742; 1997.
53. Tobiasch, E.; Gunther, L.; Bach, F. H. Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. *J. Investig. Med.* 49(6):566–571; 2001.
54. Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care* 27(5):1047–1053; 2004.
55. Wolff, S. P. Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. *Br. Med. Bull.* 49(3):642–652; 1993.
56. Yagishita, Y.; Fukutomi, T.; Sugawara, A.; Kawamura, H.; Takahashi, T.; Pi, J.; Uruno, A.; Yamamoto, M. Nrf2 protects pancreatic beta-cells from oxidative and nitrosative stress in diabetic model mice. *Diabetes* 63(2):605–618; 2014.
57. Yates, M. S.; Tauchi, M.; Katsuoka, F.; Flanders, K. C.; Liby, K. T.; Honda, T.; Gribble, G. W.; Johnson, D. A.; Johnson, J. A.; Burton, N. C.; Guilarte, T. R.; Yamamoto, M.; Sporn, M. B.; Kensler, T. W. Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol. Cancer Ther.* 6(1):154–162; 2007.
58. Zoja, C.; Corna, D.; Nava, V.; Locatelli, M.; Abbate, M.; Gaspari, F.; Carrara, F.; Sangalli, F.; Remuzzi, G.; Benigni, A. Analogs of bardoxolone methyl worsen diabetic nephropathy in rats with additional adverse effects. *Am. J. Physiol. Renal Physiol.* 304(6):F808–819; 2013.