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Deficiency in the nuclear factor E2-related factor 2 renders pancreatic β -cells vulnerable to arsenic-induced cell damage

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Abstract

Chronic human exposure to inorganic arsenic (iAs), a potent environmental oxidative stressor, is associated with increased prevalence of Type 2 diabetes, where impairment of pancreatic β -cell function is a key pathogenic factor. Nuclear factor E2-related factor 2 (Nrf2) is a central transcription factor regulating cellular adaptive response to oxidative stress. However, persistent activation of Nrf2 in response to chronic oxidative stress, including inorganic arsenite (iAs³⁺) exposure, blunts glucose-triggered reactive oxygen species (ROS) signaling and impairs glucose-stimulated insulin secretion (GSIS). In the current study, we found that MIN6 pancreatic β -cells with stable knockdown of *Nrf2* (*Nrf2*-KD) by lentiviral shRNA and pancreatic islets isolated from *Nrf2*-knockout (*Nrf2*^{-/-}) mice exhibited reduced expression of several antioxidant and detoxification enzymes in response to acute iAs³⁺ exposure. As a result, *Nrf2*-KD MIN6 cells and *Nrf2*^{-/-} islets were more susceptible to iAs³⁺ and monomethylarsonous acid (MMA³⁺)-induced cell damage, as measured by decreased cell viability, augmented apoptosis and morphological change. Pretreatment of MIN6 cells with Nrf2 activator tert-butylhydroquinone protected the cells from iAs³⁺-induced cell damage in an Nrf2-dependent fashion. In contrast, antioxidant N-acetyl cysteine protected *Nrf2*-KD MIN6 cells against acute cytotoxicity of iAs³⁺. The present study demonstrates that Nrf2-mediated antioxidant response is critical in the pancreatic β -cell defense mechanism against acute cytotoxicity by arsenic. The findings here, combined with our previous results on the inhibitory effect of antioxidants on ROS signaling and GSIS, suggest that Nrf2 plays paradoxical roles in pancreatic β -cell dysfunction induced by environmental arsenic exposure.

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Conflict of interest

The content is solely the responsibility of the authors. All authors have agreed to its content. M.E.A received some funding from DOW Chemical Company and Unilever. All of the other authors declare no competing financial interests. B.Y., J.F., H.Z., P.X., K.Y., C.G.W., Y.H., Q.Z., M.E.A. and J.P. are employees of The Hamner Institutes for Health Sciences. The Hamner is a 501(c)3 non-profit organization that has a diverse research portfolio including funding from the American Chemistry Council, a trade association that represents chemical manufacturers.

Keywords

arsenic; diabetes; pancreatic β -cell; islets; Nrf2; oxidative stress; cytotoxicity

Introduction

There is growing evidence that chronic exposure to inorganic arsenic (iAs) is associated with increased prevalence of Type 2 diabetes (T2D), a metabolic disease that is attributed to a combination of genetic, lifestyle and environmental factors (Lai *et al.*, 1994; Rahman *et al.*, 1998; Wang *et al.*, 2003; Tseng, 2004; Nabi *et al.*, 2005; Chiu *et al.*, 2006; Coronado-Gonzalez *et al.*, 2007; Meliker *et al.*, 2007; Navas-Acien *et al.*, 2008; Navas-Acien *et al.*, 2009; Del Razo *et al.*, 2011). However, the mode of action for the diabetogenic effect of iAs is largely unknown. The development of T2D is underpinned by a progressive worsening of insulin responsiveness and pancreatic β -cell function (Lowell and Shulman, 2005; Robertson and Harmon, 2006). Normal pancreatic β -cells can compensate for attenuated insulin sensitivity by increasing insulin secretion and/or β -cell mass (Prentki and Nolan, 2006). Insufficient compensation ultimately leads to the onset of glucose intolerance and T2D (Kajimoto and Kaneto, 2004). Amidst the various mechanisms proposed for β -cell dysfunction and their roles in the progression to diabetes, oxidative stress is a common denominator (Evans *et al.*, 2003; Kajimoto and Kaneto, 2004; Robertson and Harmon, 2006).

Nuclear factor E2-related factor 2 (Nrf2, also known as Nfe2l2) is a master regulator of the cellular adaptive response to oxidative and electrophilic stress (Maher and Yamamoto, 2010; Pi *et al.*, 2010b). In response to oxidative/electrophilic stress, Nrf2 heterodimerizes with small Maf proteins and other basic leucine zipper proteins, binding to antioxidant response elements (AREs) in the promoters of many phase II detoxification and antioxidant genes to increase their transcription (Maher and Yamamoto, 2010). Evidence supporting the pivotal roles of Nrf2 in protecting against oxidative/electrophilic stress comes, in part, from studies conducted in *Nrf2*-knockout (*Nrf2*^{-/-}) mice and *Nrf2*-knockdown (*Nrf2*-KD) cells. These animals and cells exhibit a severe deficiency in the coordinated gene regulatory program for adaptive antioxidant response, resulting in a high susceptibility to oxidative stress-related disorders, chemical carcinogenesis and cytotoxicity (Itoh *et al.*, 2010; Liu *et al.*, 2010; Zhao *et al.*, 2012). Thus, the Nrf2-mediated antioxidant response represents a critically important cellular defense mechanism that serves to maintain intracellular redox homeostasis and limit oxidative damage. iAs and its methylated trivalent metabolites, including monomethylarsonous acid (MMA³⁺) and dimethylarsinous acid (DMA³⁺), are potent oxidative stressors (Pi *et al.*, 2002; Pi *et al.*, 2003a; Pi *et al.*, 2003b; Bailey *et al.*, 2010). Acute exposure to inorganic arsenite (iAs³⁺) and MMA³⁺ has been demonstrated to activate Nrf2-mediated adaptive antioxidant response in a variety of human and rodent cells (Pi *et al.*, 2003b; Pi *et al.*, 2007a; Pi *et al.*, 2008; Wang *et al.*, 2008; Zhao *et al.*, 2011; Zhao *et al.*, 2012). Our previous studies (Pi *et al.*, 2007b; Fu *et al.*, 2011) reported that up to 96 hrs of exposure of pancreatic β -cell line INS-1(832/13) cells and isolated mouse islets to low levels of iAs³⁺ led to decreased glucose-stimulated insulin secretion (GSIS), which is associated with adaptive induction of antioxidant enzymes, including catalase and glutathione peroxidase. However, the precise role of Nrf2 in arsenic-induced β -cell dysfunction is still unclear. Here we report that Nrf2-mediated antioxidant response plays a critical role in the pancreatic β -cell defense mechanism against the acute cytotoxicity induced by high-concentrations of iAs³⁺ and MMA³⁺. Given the important role of reactive oxygen species (ROS) signaling in GSIS (Pi *et al.*, 2007b), the present study and our previous findings on the inhibitory effect of antioxidants on ROS signaling (Pi *et al.*, 2007b; Fu *et al.*, 2011)

suggest that Nrf2 plays paradoxical roles in pancreatic β -cell dysfunction induced by environmental arsenic exposure.

Materials and Methods

Cell culture and reagents

MIN6 cells were kindly provided by Dr. Marcia Haigis (Harvard University, Boston, MA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, with 15% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 5 μ L β -mercaptoethanol in humidified 5% CO₂, 95% air at 37°C. Culture media, FBS, phosphate buffered saline (PBS, pH7.4) and supplements were purchased from Invitrogen (Carlsbad, CA). Sodium arsenite, β -mercaptoethanol, *tert*-Butylhydroquinone (tBHQ), sulforaphane (SFN) and N-acetyl cysteine (NAC) were obtained from Sigma (St. Louis, MO). Methylarsine oxide (CH₃AsO) was synthesized by Dr. William R. Cullen (University of British Columbia, Vancouver, Canada) using a method described previously (Cullen *et al.*, 1989; Petrick *et al.*, 2001).

Lentiviral-based shRNA transduction

MISSION shRNA lentiviral vectors were obtained from Sigma and lentiviral particles were prepared using the manufacturer's protocol. Lentiviral transduction of MIN6 cells with particles for shRNAs targeting Nrf2 (SHVRSNM_010902) or Scrambled (Scr) non-target negative control (SHC002V) was performed as described previously (Pi *et al.*, 2010a). Briefly, 24 hrs prior to transduction, MIN6 cells were plated in 6-well plates at 40–50% confluency in complete medium described above. The following day, hexadimethrine bromide (Sigma), a transduction enhancer, was added to each well at a concentration of 8 μ g/ml and viral particles were added to each well at a concentration of 2×10^5 transducing units (TU) per ml. Following a 24-hr incubation period, medium containing viral particles was removed and replaced with fresh medium containing 1 μ g/ml of puromycin. Cells were grown to 90% confluence and sub-cultured in medium containing puromycin (1 μ g/m).

Animals

Nrf2^{-/-} mice developed as described previously (Itoh *et al.*, 1997) were kindly provided by Dr. Masayuki Yamamoto (Tohoku University, Japan). The mice were backcrossed onto the C57BL/6J background for 7 generations using alternating male and female stock mice from The Jackson Laboratories (Bar Harbor, ME). The resulting wild-type (*Nrf2*^{+/+}) and *Nrf2*^{-/-} mice were used in the present study. The mice were housed in virus-free facilities on a 12-hr light/12-hr dark cycle and were fed NIH07 chow diet (Zeigler Brothers Gardners, PA) and provided with reverse osmosis water *ad libitum*. Genotyping was performed by PCR (primer sequences in Online Material Table S1) using genomic DNA that was isolated from tail snips. All protocols for animal use were approved by the Institutional Animal Care and Use Committee of The Hamner Institutes and were in accordance with the National Institutes of Health guidelines.

Islet isolation and primary culture

Pancreatic islets were isolated from 9–12 week-old mice by collagenase P (Roche, Switzerland) digestion, as described previously (Pi *et al.*, 2007b). Islets were cultured in RPMI 1640 supplemented with 10 mM glucose, 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The islets were cultured for 48 hrs before arsenic treatment.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA in MIN6 cells was isolated with TRIzol reagent (Invitrogen) and subsequently subjected to cleanup using an RNase-Free DNase Set and RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA in cultured islets (500 islets each condition) was isolated using the RNeasy Mini Kit. Total RNA was reverse transcribed with MuLV reverse transcriptase and Oligo d(T) primers (Applied Biosystems, Foster City, CA). A SensiFAST SYBR Hi-ROX kit (BIOLINE USA Inc, Taunton, MA) was used for qPCR. The primers were designed using Primer Express 4 (Applied Biosystems) and synthesized by Bioneer Inc. (Alameda, CA). The primer sequences are listed in Table S3. Relative differences in gene expression among groups were determined from quantification cycle (C_q) values. These values were first normalized to 18 ribosomal RNA (18S) in the same sample (ΔC_q) and expressed as the fold-change over control ($2^{-\Delta\Delta C_q}$). Real-time fluorescence detection was performed using an ABI PRISM 7900HT Fast Real-time PCR System (Applied Biosystems). Details on the procedures of RNA quantification and RT-qPCR are described in Online Supplemental Materials.

Western blot analysis

Isolation of cell fractions and immunoblotting were performed as detailed previously (Pi *et al.*, 2003b; Woods *et al.*, 2009). An antibody for Nrf2 (sc-13032; 1:500) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for caspase-3 (#9662, 1: 1000) and cleaved caspase 3 (#9661, 1:1000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against β -ACTIN (A1978; 1:2000) and LAMIN A (L1293; 1:2500) were from Sigma.

Measurement of cell viability and caspase-3/7 activity

Cell viability was determined by using Non-Radioactive Cell-Proliferation Assay Kit (Promega, Madison, WI) as detailed previously (Pi *et al.*, 2005). Caspase-3/7 activity was measured using the ApoLive-Glo Multiplex Assay Kit (Promega, Madison, WI) per the manufacturer's recommendation. A minimum of 5 replicates of 10,000 cells per well were plated in 96-well plates and allowed to adhere to the plate for 24 hrs, after which the media was removed and replaced with fresh media containing various concentrations of arsenicals. Cells were subsequently incubated for an additional 24 hrs, and cell viability and caspase-3/7 activity were determined.

Cell death assessment by flow cytometry

MIN6 cells were seeded in a 6-well plate and grown to approximately 80% confluence. After the cells were treated with iAs^{3+} for 24 hrs, the floating and attached cells were harvested for apoptosis analysis. Apoptotic and necrotic cells were analyzed by flow cytometry (FACS CantoII with HTS option, BD Biosciences) using the TACS Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) as detailed previously (Pi *et al.*, 2005). For each sample, 10,000 cells were examined. The percentage of apoptotic and necrotic cells was determined by statistical analysis of the various dot plots by using CellQuest software (Diva 6.0, BD Biosciences).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining

TUNEL staining of islets was performed using an ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (EMD Millipore, Billerica, MA). Briefly, following a 48-hr culture period, isolated islets were treated with iAs^{3+} for 24 hrs. Subsequently, the islets were washed with PBS and fixed for 24 hrs at room temperature in 2% (v/v) formaldehyde. After washing in PBS, islets were embedded in paraffin, sectioned, de-paraffinized and re-

hydrated as described previously (Pi *et al.*, 2009). The slides were then treated with proteinase K (20 µg/ml) for 15 min and incubated in 3% (v/v) H₂O₂ for 5 min to quench endogenous peroxidase activity. The slides were labeled by incubating for 60 min at 37 °C in the presence of terminal deoxynucleotidyl transferase (TdT) and for another 30 min in an anti-digoxigenin antibody conjugated with horseradish peroxidase. Negative controls were also prepared in which TdT treatment was omitted. Following TUNEL staining, slides were mounted with the Prolong Gold antifade reagent with DAPI (P36931, Invitrogen) and covered with coverslips and examined using an Axio Observer Z1 fluorescence microscope (Carl Zeiss, Inc. Oberkochen, Germany).

Statistical analyses

All statistical analyses were performed by using Graphpad Prism 4 (GraphPad Software, San Diego, CA), with $p < 0.05$ taken as significant. More specific indices of statistical significance are indicated in individual figure legends. Data are expressed as mean $\pm$ SD. For comparisons between two groups, a Student's t-test was performed. For comparisons among three or more groups, one-way or two-way ANOVA with Bonferroni post hoc testing was performed.

Results

Stable knockdown of Nrf2 in MIN6 cells reduces the adaptive antioxidant response induced by iAs³⁺

In MIN6 cells, transduction of lentiviral shRNA against mouse *Nrf2* (termed *Nrf2*-KD) significantly attenuated the mRNA expression of *Nrf2* compared to Scr control cells (Fig. 1A). The effectiveness of Nrf2 silencing was confirmed by notably diminished protein expression of Nrf2 in cells challenged with known Nrf2 activators, including iAs³⁺, tBHQ and SFN (Fig. 1B). To determine the acute effect of iAs³⁺ exposure on Nrf2-mediated antioxidant response in pancreatic β -cells, the time-course (Fig. 1C and D) and concentration-response (Fig. 1E and F) of Nrf2 expression in response to iAs³⁺ exposure were determined at mRNA (Fig. 1C and E) and protein (Fig. 1D and F) levels in Scr and *Nrf2*-KD cells. In contrast to a late induction of mRNA expression of Nrf2 (Fig. 1C), the protein levels of Nrf2 were elevated quickly and peaked between 2–6 hrs in Scr cells (Fig. 1D). Importantly, in response to a 6-hr exposure to iAs³⁺, the protein expression of Nrf2 in Scr cells was increased in a concentration-dependent manner. In contrast, *Nrf2*-KD cells expressed remarkably reduced levels of Nrf2 protein under basal and iAs³⁺-exposed conditions (Fig. 1F). In addition, acute iAs³⁺ exposure led to increased protein accumulation of Nrf2 in the nuclear fractions of Scr cells in a concentration-dependent fashion. *Nrf2*-KD cells showed a substantial reduction in iAs³⁺-induced Nrf2 nuclear accumulation (Fig. 2A). Furthermore, the expression of ARE-dependent genes, including NAD(P)H quinone oxidoreductase 1 (*Nqo1*), heme oxygenase 1 (*Hmox-1*), glutamate-cysteine ligase catalytic (*Gclc*) and sulfiredoxin (*Srx*) was substantially attenuated by Nrf2 silencing, indicating that Nrf2-mediated transcription was suppressed in *Nrf2*-KD cells (Fig. 2B and C).

Silencing of Nrf2 sensitizes MIN6 cells to iAs³⁺ and CH₃AsO-induced acute cytotoxicity

To study the protective effect of Nrf2 against the cytotoxicity of arsenic in pancreatic β -cells, we investigated the effect of stable knockdown of *Nrf2* on the susceptibility to arsenic-induced cytotoxicity in MIN6 cells. As expected, *Nrf2* silencing rendered the cells more susceptible to iAs³⁺-induced reduction in cell viability (Fig. 3A). This sensitization to iAs³⁺-induced cytotoxicity was further confirmed by measurement of apoptosis and necrosis using flow cytometry with Annexin V-FITC and propidium iodide (PI) double staining (Fig. 3C and D), as well as the expression and activity of Caspase 3 and/or 7 (Fig. 3E and F). It is generally accepted that iAs, including iAs³⁺ and iAs⁵⁺, are metabolized in humans by

enzymatic and non-enzymatic mechanisms (Aposhian, 1997; Hayakawa *et al.*, 2005) into monomethylarsonous acid (MMA^{3+}), monomethylarsonic acid (MMA^{5+}), dimethylarsinous acid (DMA^{3+}) and dimethylarsinic acid (DMA^{5+}). MMA ($\text{MMA}^{3+} + \text{MMA}^{5+}$) and DMA ($\text{DMA}^{3+} + \text{DMA}^{5+}$) are the major metabolites of iAs in human blood and urine (Pi *et al.*, 2002). In addition, high levels of MMA and DMA were detected in the pancreas of mice with chronic iAs exposure (Paul *et al.*, 2011), suggesting that methylated metabolites of iAs may be involved in environmental iAs exposure-induced β -cell dysfunction. Thus, the cytotoxic effect of MMA^{3+} was determined in MIN6 cells. As shown in Fig. 3B, CH_3AsO exhibited much more of a potent inhibitory effect on cell viability than iAs^{3+} in MIN6 cells. In particular, *Nrf2*-KD cells exhibited significantly higher sensitivity than Scr to CH_3AsO -induced cytotoxicity.

***Nrf2*^{-/-} islets show abrogated antioxidant response and are more sensitive to iAs^{3+} -induced acute cytotoxicity**

To validate the major findings in MIN6 cells, the *Nrf2*-mediated antioxidant response and cytotoxicity induced by acute iAs^{3+} exposure were determined in isolated mouse islets. As shown in Fig. 4A, acute iAs^{3+} exposure increased the expression of *Nrf2* and its downstream genes, including *Nqo1*, *Hmox-1*, *Gclc* and *Srx*, in *Nrf2*^{+/+} islets. Deficiency of *Nrf2* resulted in significantly reduced expression of most of these ARE-dependent genes. Following a 24-hr iAs^{3+} (20 or 50 μM) exposure, many islets lost their intact membranes and dispersed into disassociated cells and/or cell debris (Fig. 4B and C), indicating the cytotoxicity of iAs^{3+} . Compared with *Nrf2*^{+/+}, *Nrf2*^{-/-} islets exhibited substantially reduced amounts of intact islets left after iAs^{3+} exposure (Fig. 4D). In addition, TUNEL staining demonstrated that *Nrf2*^{-/-} islets were more sensitive to iAs^{3+} -induced apoptosis (Fig. 5).

***Nrf2* activation protects MIN6 cells against acute iAs^{3+} cytotoxicity in an *Nrf2*-dependent fashion**

To provide further support of our hypothesis that *Nrf2* activation protects pancreatic β -cells against iAs^{3+} cytotoxicity, the effect of pretreatment of MIN6 cells with *Nrf2* activator tBHQ on iAs^{3+} -induced cytotoxicity was examined. As shown in Fig. 6A, pretreatment of Scr cells with non-cytotoxic levels of tBHQ (50 μM) for 6 hrs showed a modest but statistically significant protection against subsequent iAs^{3+} toxicity. However, pretreatment of *Nrf2*-KD cells with tBHQ showed no protective effect (Fig. 6B), suggesting that the protective effect of tBHQ pretreatment against iAs^{3+} toxicity is a result of *Nrf2* activation. Interestingly, NAC showed a significant protective effect from iAs^{3+} toxicity in *Nrf2*-KD cells, suggesting that exogenous antioxidants may rescue the impairment of the cellular antioxidant system caused by *Nrf2* deficiency.

Discussion

Diabetes is a chronic disease characterized by high levels of glucose in the blood. There are four clinical classes, including Type 1 diabetes (T1D), T2D, gestational diabetes and other specific types of diabetes due to other causes. T1D results from autoimmune pancreatic β -cell destruction, usually leading to absolute insulin deficiency (Atkinson *et al.*, 2011), whereas T2D results from a progressive insulin secretion defect on the background of insulin resistance (Robertson, 2006). While the precise mechanisms for the diabetogenic effect of iAs are still largely undefined, recent *in vitro* and *in vivo* experimental studies indicated that iAs or its metabolites impair insulin-dependent glucose uptake and result in insulin resistance (Izquierdo-Vega *et al.*, 2006; Paul *et al.*, 2007a; Paul *et al.*, 2007b; Paul *et al.*, 2011; Xue *et al.*, 2011). In addition, accumulating epidemiological studies demonstrate that insulin resistance is a common symptom in diabetes associated with chronic iAs

exposure (Lai *et al.*, 1994; Rahman *et al.*, 1998; Wang *et al.*, 2003; Tseng, 2004; Nabi *et al.*, 2005; Chiu *et al.*, 2006; Coronado-Gonzalez *et al.*, 2007; Meliker *et al.*, 2007; Navas-Acien *et al.*, 2008; Navas-Acien *et al.*, 2009; Del Razo *et al.*, 2011). Thus, T2D is the most predominant, if not only, type of diabetes associated with iAs exposure. It is clear that insulin resistance plays an early pathogenic role in the development of T2D and defects in insulin secretion by pancreatic β -cells are instrumental in the progression to hyperglycemia (Lowell and Shulman, 2005). In isolated rodent islets and cultured β -cells, iAs was found to decrease insulin transcription and GSIS (Diaz-Villasenor *et al.*, 2006; Pi *et al.*, 2007b; Fu *et al.*, 2011). In the present study, we found that iAs^{3+} and CH_3AsO , a form of MMA^{3+} , induce apoptosis and/or cytotoxicity in β -cell line MIN6 cells and isolated mouse islets in a concentration- and time-dependent fashion. These findings suggest that β -cell damage and the associated dysfunction in insulin synthesis and secretion may be an important contributing factor for iAs-induced diabetes.

Although the precise molecular mechanisms of β -cell dysfunction in T2D are not completely understood, oxidative stress has been increasingly implicated in the pathogenesis of progressive β -cell failure (Evans *et al.*, 2003; Kajimoto and Kaneto, 2004; Robertson and Harmon, 2006). It has been well documented that excessive and/or sustained increases in ROS production can directly or indirectly disturb the physiological function of cellular macromolecules such as DNA, protein or lipids and activate cellular stress-sensitive signaling pathways (Evans *et al.*, 2003). In the early stages of oxidative stress, the adaptive response, primarily regulated by Nrf2, is the main response that will upregulate antioxidant and phase II detoxification enzymes and protect cells from more serious oxidative damage for cell survival. In the absence of an appropriate compensatory response from the endogenous antioxidant network, oxidative stress may cause oxidative damage and activate the cell death machinery (Davies, 2000). In this regard, abolishment of the Nrf2-mediated antioxidant response by targeted disruption of the Nrf2 gene in β -cell line MIN6 cells and mouse islets increased the susceptibility to iAs^{3+} and MMA^{3+} -induced cytotoxicity and/or apoptosis. In contrast, activation of Nrf2 with tBHQ pretreatment significantly protects MIN6 cells from iAs^{3+} -induced acute cytotoxicity in an Nrf2-dependent manner. These findings suggest that Nrf2 plays a critical role in pancreatic β -cell defense against iAs^{3+} and MMA^{3+} -induced oxidative/electrophilic stress.

Although potentially cytotoxic, ROS also function as important intracellular signaling molecules for cellular responses to a variety of physiological stimuli, including glucose sensing in pancreatic β -cells (Pi *et al.*, 2007b; Pi *et al.*, 2010b). The involvement of ROS as signaling intermediates suggests that their magnitude would be inversely correlated with the ROS-scavenging activity and antioxidant status in the cell. Therefore, we propose that Nrf2-mediated antioxidant response plays a paradoxical role in arsenic-induced pancreatic β -cell dysfunction. On the one hand, it protects β -cells from high-dose arsenic-induced oxidative damage and possible cell death, thus minimizing oxidative damage-related impairment of insulin secretion. This premise was demonstrated by the present study indicating that Nrf2-mediated antioxidant response protects pancreatic β -cells from acute cytotoxicity of iAs^{3+} and MMA^{3+} . On the other hand, because ROS signaling triggered by glucose could be an important component driving insulin secretion, the induction of endogenous antioxidants in the presence of iAs^{3+} and/or its methylated metabolites may blunt the ROS signaling resulting in reduced GSIS. Thus, we envision the following scenarios for arsenic-induced impairment of β -cell function. Under low, environmentally relevant levels of arsenic exposure, β -cells can adapt to the condition adequately by activating the Nrf2-ARE system, thereby keeping oxidative damage/cell death-related impairment of GSIS at a minimum. However, under chronic exposure conditions, the adaptively increased endogenous antioxidant capacity may interfere with glucose-dependent ROS signals that we postulate directly contribute to GSIS, leading reversibly to attenuated GSIS. Under high-dose arsenic

exposure, the Nrf2 system may protect the cells from irreversible oxidative damage and possible cell death, which may be the primary cause for impaired GSIS under high-dose conditions. Thus the machinery of GSIS in β -cells is likely to be highly sensitive to arsenic exposure, though distinct mechanisms may apply through the dose continuum. These considerations are compatible with the view that oxidative stress may contribute to both early and late phases of β -cell failure in T2D.

In summary, the present study demonstrated that a deficiency in Nrf2-mediated antioxidant response renders pancreatic β -cells vulnerable to arsenic-induced cell damage, whereas activation of Nrf2 protects β -cells from arsenic toxicity. Considering the likely inhibitory effect of persistent Nrf2 activation on ROS signaling in GSIS, the potential application of activating Nrf2 to prevent and/or treat T2D associated with arsenic exposure should be approached with caution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

18S	18S ribosomal RNA
ARE	antioxidant response element
DMA³⁺	dimethylarsinous acid
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
GCLC	γ -glutamate cysteine ligase catalytic subunit
GSIS	glucose-stimulated insulin secretion
Hmox1	heme oxygenase 1
iAs	inorganic arsenic
iAs³⁺	inorganic arsenite
MMA³⁺	monomethylarsonous acid
Nqo1	NAD(P)H: quinone oxidoreductase 1
Nrf2	Nuclear factor E2-related factor 2
<i>Nrf2</i>^{-/-}	<i>Nrf2</i> -knockout
<i>Nrf2</i>-KD	<i>Nrf2</i> -knockdown
PBS	phosphate buffered saline
PI	propidium iodide
ROS	reactive oxygen species

SFN	sulforaphane
Srx	sulfiredoxin 1
tBHQ	<i>tert</i> -Butylhydroquinone
T1D	Type 1 diabetes
T2D	Type 2 diabetes

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Highlights

- Lack of Nrf2 reduced expression of antioxidant genes induced by iAs³⁺ in β -cells.
- Deficiency of Nrf2 in β -cells sensitized to iAs³⁺ and MMA³⁺-induced cytotoxicity
- Nrf2 activation protected β -cells from acute iAs³⁺ cytotoxicity

Fig. 1.

Stable knockdown of *Nrf2* results in reduced expression of Nrf2 in MIN6 cells. (A) mRNA expression of *Nrf2* in MIN6 cells transduced with shRNA lentivirus targeted against mouse *Nrf2* or Scrambled non-target negative control (Scr). (B) Reduced protein expression of Nrf2 in *Nrf2*-KD cells under basal and iAs^{3+} (5 μ M), *tert*-Butylhydroquinone (tBHQ, 50 μ M) or sulforaphane (SFN, 10 μ M)-treated conditions. Cells were treated with the chemicals for 6 hrs. Whole cell lysates were used for immunoblotting, and β -ACTIN was used as a loading control. Vehicle, medium. (C and D) Time course of iAs^{3+} -induced Nrf2 mRNA (C) and protein (D) expression. Cells were exposed to 4 μ M iAs^{3+} for the indicated time. (E and F) Concentration-response of iAs^{3+} -induced Nrf2 mRNA (E) and protein (F) expression after cells were exposed to iAs^{3+} for 6 hrs. Values in A, C and E are mean $\pm$ SD. $n = 3-6$. * $p < 0.05$ vs. Scr with the same treatment.

Fig. 2.

Nrf2 silencing results in reduced antioxidant response induced by iAs³⁺ in MIN6 cells. (A) Nrf2 protein levels in nuclear fractions of MIN6 cells. Lamin A was used as a loading control. (B and C) Lack of *Nrf2* significantly reduces the expression of ARE-dependent genes. The concentration of iAs³⁺ used in B is 5 μ M. The time point at which the measurements were made in C is 6 hrs. The expression of *Nqo1*, *Hmox-1*, *Gclc* and *Srx* was measured by real-time RT-PCR. The number in brackets following the gene name is the Cq value of that gene in Scr cells with vehicle. Values in B and C are means $\pm$ SD. n = 3. **p* < 0.05 vs. Scr with the same treatment.

Fig. 3.

Stable knockdown of *Nrf2* increases the susceptibility of MIN6 cells to iAs^{3+} - and MMA^{3+} -induced cytotoxicity. (A and B) *Nrf2*-KD cells are more sensitive to the cytotoxicity of iAs^{3+} (A) and MMA^{3+} (B). Cell viability was assessed by ApoLive-Glo Multiplex Assay following a 24-hr treatment with the indicated concentrations of arsenicals. (C) Representative plots of apoptosis measurements by flow cytometry. MIN6 cells stained with Annexin V and PI before being analyzed. FL, fluorescence. (D) Quantification of iAs^{3+} -induced apoptosis by flow cytometry. Cells were exposed to iAs^{3+} for 24 hrs. Annexin V-positive cells were quantified as apoptotic cells. (E) Immunoblotting of Caspase-3 and cleaved-Caspase-3. Cells were exposed to iAs^{3+} for 24 hrs. Whole cell lysates were used for

analysis, and β -ACTIN was used as a loading control. Vehicle, medium. (F) The activity of Caspase 3/7 measured by the Apolive-Glo Multiplex Assay in MIN6 cells exposed to iAs^{3+} for 24 hrs. Values in A, B, D and F are means $\pm$ SD. n = 3–6. * $p < 0.05$ vs. Scr with the same treatment.

Fig. 4.

Deficiency of Nrf2 results in reduced antioxidant response and increased sensitivity to acute iAs³⁺ exposure-induced cytotoxicity in primary cultured mouse islets. (A) *Nrf2*^{-/-} islets exhibit reduced expression of ARE-dependent genes. Isolated islets were exposed to 5 μM iAs³⁺ for 6 hrs followed by immediate isolation of total RNA and real-time RT-PCR measurement for *Nqo1*, *Hmox-1*, *Gclc* and *Srx*. The number in brackets following the gene name is the Cq value of that gene in *Nrf2*^{+/+} islets with Vehicle. n = 3. **p* < 0.05 vs. the same genotype with vehicle; #*p* < 0.05 vs. *Nrf2*^{+/+} islets with the same treatment. (B) Representative images of cultured islets following a 24-hr treatment with indicated concentrations of iAs³⁺. n = 3. The islets were examined by the Olympus SZX7 Zoom Stereomicroscope (4 ×). (C) Representative images of cultured islets following a 24-hr iAs³⁺ (10 μM) treatment. The islets were examined by the Carl Zeiss Axiovert 40 CFL inverted microscope (10 ×). (D) Quantification of intact islets that remained following a 24-hr iAs³⁺ exposure as in (B).

Fig. 5.

(A) Representative TUNEL staining in cultured islets which were incubated for 24 h in the presence or absence of 10 μ M of iAs³⁺, red = DAPI; green = TUNEL staining. (B) Quantification of TUNEL-positive cells in islets. n = 15 islets for each condition. * $p < 0.05$ vs. *Nrf2*^{+/+} islets with the same treatment. # $p < 0.05$ vs. *Nrf2*^{+/+} islets with iAs³⁺ treatment.

Fig. 6.

(A and B) Effect of pretreatment with non-cytotoxic levels of tBHQ on iAs^{3+} cytotoxicity in Scr (A) and *Nrf2*-KD (B) MIN6 cells. Cells were pretreated with 50 μM tBHQ for 6 hrs followed by a 24-hr iAs^{3+} exposure. (C and D) Effect of NAC on the cytotoxicity of iAs^{3+} in Scr (C) and *Nrf2*-KD (D) MIN6 cells. Cell viability was measured using the Non-Radioactive Cell-Proliferation Assay Kit. $n = 6$. *, $p < 0.05$ vs. Non-pretreated cells with the same concentration of iAs^{3+} .