RESEARCH PAPER

Divergent Effects of Sulforaphane on Basal and Glucose-Stimulated Insulin Secretion in β-Cells: Role of Reactive Oxygen Species and Induction of Endogenous Antioxidants

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ABSTRACT

Purpose Oxidative stress is implicated in pancreatic β-cell dysfunction, yet clinical outcomes of antioxidant therapies on diabetes are inconclusive. Since reactive oxygen species (ROS) can function as signaling intermediates for glucose-stimulated insulin secretion (GSIS), we hypothesize that exogenously boosting cellular antioxidant capacity dampens signaling ROS and GSIS.

Methods To test the hypothesis, we formulated a mathematical model of redox homeostatic control circuit comprising known feedback and feedforward loops and validated model predictions with plant-derived antioxidant sulforaphane (SFN).

Results SFN acutely (30-min treatment) stimulated basal insulin secretion in INS-1(832/13) cells and cultured mouse islets, which could be attributed to SFN-elicited ROS as N-acetylcysteine or glutathione ethyl ester suppressed SFN-stimulated insulin secretion. The mathematical model predicted an adapted redox state characteristic of strong induction of endogenous antioxidants but marginally increased ROS under prolonged SFN exposure, a state that attenuates rather than facilitates glucose-stimulated ROS and GSIS. We validated the prediction by demonstrating that although 24-h treatment of INS-1(832/13) cells with low, non-cytotoxic concentrations of SFN (2–10 μM) protected the cells from cytotoxicity by oxidative insult, it markedly suppressed insulin secretion stimulated by 20 mM glucose.

Conclusions Our study indicates that adaptive induction of endogenous antioxidants by exogenous antioxidants, albeit cytoprotective, inhibits GSIS in β-cells.

KEY WORDS antioxidant · computational model · insulin secretion · Nrf2 · ROS · sulforaphane

ABBREVIATIONS

AC	Antioxidant capacity
ARE	Antioxidant response element
BSA	Bovine serum albumin
CM-H2DCFDA	5-(and-6)-chloromethyl-2' 7'-dichlorodihydrofluorescein diacetate, acetyl ester
DEM	Diethylmaleate
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
GCLC	γ-glutamate cysteine ligase catalytic subunit
GSH-EE	Glutathione ethyl ester

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GSIS   Glucose-stimulated insulin secretion
H_2O_2 Hydrogen peroxide
HMOX-1   Heme oxygenase 1
NAC   N-acetylcysteine
NQO1   NAD(P)H: quinone oxidoreductase 1
Nrf2   Nuclear factor erythroid 2-related factor 2
ROS   Reactive oxygen species
SFN   Sulforaphane
SRXN1   Sulfiredoxin 1
T1D   Type 1 diabetes
T2D   Type 2 diabetes
tBHQ   Tert-butylhydroquinone

INTRODUCTION

Insulin-producing pancreatic β-cells are the most abundant and important cell type in the islet of Langerhans. Autoimmune destruction of β-cells with consequent absolute insulin deficiency results in Type 1 Diabetes (T1D) (1). The development of Type 2 Diabetes (T2D) is usually associated with a combination of insulin resistance and pancreatic β-cell dysfunction involving relative insulin deficiency (2,3). Therefore, impairment of pancreatic β-cell function is a primary contributor to the development of both types of diabetes. Although the precise pathogenic mechanisms of β-cell dysfunction are not completely understood, there is compelling evidence that oxidative stress, as a result of human exposure to environmental and dietary factors, plays a prominent role (1–5).

If unchecked, oxidative stress can considerably increase intracellular reactive oxygen species (ROS) levels, leading to oxidative damage to proteins, nucleic acids and fatty acids. Oxidative damage is believed to be a major contributing factor to the development of pancreatic β-cell dysfunction (6) and of insulin resistance in the adipose tissue, liver and skeletal muscle (7). As a result, antioxidant supplementation has been arduously advocated as a preventive and therapeutic measure to combat oxidative stress implicated in diabetes as well as a variety of other diseases. However, clinical antioxidant therapy is not as effective as expected for many disease endpoints including diabetes (8–17). While the observed ineffectiveness of antioxidant supplements may be attributed to many factors, including trial design, dose, frequency and bioavailability, these clinical data cast serious doubt on their usefulness.

Although cytotoxic at higher levels, ROS also function as intracellular second messengers. For instance, ROS derived from glucose metabolism act as a necessary intermediate signal to mediate glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells and glucose sensing in hypothalamic neurons (18–23). The involvement of ROS as a signaling intermediate suggests that its magnitude would be inversely correlated with the ROS-scavenging capacity in the cell. This notion conceivably creates a conundrum for the effect of exogenous antioxidants on β-cell function. On the one hand, exogenous antioxidants can be cytoprotective against oxidative damage. On the other hand, they may indiscriminately attenuate glucose-stimulated ROS accumulation and thus GSIS. The latter may well explain why antioxidant supplementation has largely failed in preventing and treating diabetes, especially given that antioxidant supplements are often taken at doses several times higher than recommended (24).

Sulforaphane (SFN) is a dietary isothiocyanate from cruciferous vegetables, with an especially high content in broccoli (25). SFN has been proven as an effective chemopreventive agent (26,27). In β-cells, SFN can protect against cytokine- and streptozotocin-induced cell damage (28). Chemically as weak pro-oxidant, SFN has been demonstrated to promote ROS generation in several cell types (29–31) and induce expression of multiple antioxidant and phase II enzymes through activating the canonical nuclear factor E2-related factor 2 (Nrf2) pathway (25,32). Because SFN and other similar phytochemical antioxidants can increase both intracellular ROS and antioxidant capacity (33,34), their effects on physiological second messenger ROS and insulin secretion are not straightforward, which may depend on the dynamical response of the cellular redox homeostatic system.

The redox homeostatic circuit is generally structured as a negative feedback loop comprising ROS, sensor protein Keap1, transcription factor Nrf2 and antioxidant enzymes (35). Indirect antioxidant phytochemicals such as SFN are able to directly modify cysteine residues of the sensor protein Keap1 to activate Nrf2 (36–38), thus forming an important feedforward loop that induces endogenous antioxidants (Fig. 1). Under chronic exposure to exogenous Nrf2-activating phytochemicals such as SFN, the redox system will adapt and settle to a steady state. We propose that due to the nonlinearity and signal amplification associated with the feedforward and feedback regulation, the steady-state antioxidant capacity would generally rise to a great extent, and as a result, the increase in basal ROS is
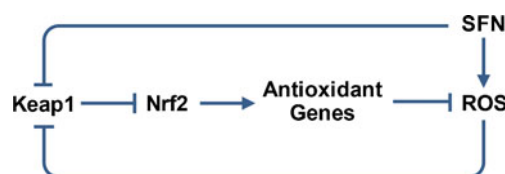
Fig. 1 A general molecular circuit controlling cellular redox homeostasis in response to perturbation by SFN. The ROS level is regulated through both negative feedback (*lower*) and incoherent feedforward (*upper*) loops. The ubiquitination activity of Keap1 is inhibited by ROS and SFN, allowing stabilization of Nrf2. Nrf2 then induces antioxidant genes, which collectively function to eliminate ROS.

very limited (39). This adapted redox state is likely to favor attenuation rather than facilitation of glucose-stimulated ROS signals and thus GSIS (35). In the present study, we formulated a mathematical model of a simplified redox homeostatic circuit to formally test the hypothesis. We verified the model predictions with SFN as a prototype antioxidant in INS-1(832/13) cells and isolated mouse islets. We found, as the model predicts, that SFN acutely stimulates basal insulin secretion in pancreatic β-cells, which can be attributed to initially uncompensated ROS production. In contrast, prolonged SFN treatment only has a marginal stimulatory effect on basal insulin secretion. More importantly, prolonged SFN treatment activates Nrf2 and the adaptive antioxidant response, which despite protecting the cells from oxidative insult, suppresses GSIS.

MATERIALS AND METHODS

Cell Culture and Reagents

INS-1(832/13) cells were kindly provided by Dr. Christopher Newgard (Duke University) and were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), 10 mM glucose, 25 mM HEPES, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Passages 55–59 at 75–90% confluence were used for the current study. There was no difference in the glucose responsiveness of the cells among different passages. Culture media, fetal bovine serum (FBS), supplements and TRIzol were purchased from Invitrogen (Carlsbad, CA). SFN, fatty acid-free bovine serum albumin (BSA), β-mercaptoethanol, glucose oxidase (GO), diethylmaleate (DEM), N-acetylcysteine (NAC), glutathione ethyl ester (GSH-EE), *tert*-Butylhydroquinone (tBHQ), potassium chloride (KCl) and glucose solution (45%) were obtained from Sigma (St. Louis, MO).

Islets Isolation and Primary Culture

Pancreatic islets were isolated from 9 to 12-week-old C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME) by collagenase P (Roche, Switzerland) digestion, as previously described (21). Islets were picked by hand four times in succession under a dissecting microscope and cultured 48 h in RPMI 1640 supplemented with 10 mM glucose, 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. All procedures were performed in accordance with the Institutional Guidelines for Animal Care at The Hamner Institutes for Health Sciences.

Measurement of Insulin Secretion

Insulin secretion in INS-1(832/13) cells and isolated islets was performed in static incubation as described previously (40). Insulin contents in the media were determined using an RIA kit (Linco Research, St. Charles, MO) with rat insulin as the standard. Levels of secreted insulin by INS-1(832/13) cells were normalized to DNA content, which was determined by an overnight incubation at 42°C with a lysis buffer containing 30 mM Tris–HCl, 10 mM EDTA, 1% SDS and 50 µg/ml proteinase K (Qiagen, Valencia, CA), followed by a measurement of absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Inc., Waltham, MA).

Intracellular Peroxide Determination

Intracellular peroxide levels in INS-1(832/13) cells were measured by flow cytometry (Becton Dickinson FACSCanto II, Becton Dickinson, San Jose, CA) using the fluorescent probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA, Invitrogen) as described previously (21). The final concentration of CM-H$_2$DCFDA used was 2 µM and loading time was 30 min.

Antioxidant Response Element Reporter Assay

Cignal Lenti antioxidant response element (ARE) reporter lentiviral particles were obtained from SABiosciences (Frederick, MD). Lentiviral transduction of INS-1(832/13) cells was performed as described previously (40,41). Cells were grown to ~90% confluency and sub-cultured in medium containing 0.35 µg/ml of puromycin. The luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The luciferase activity was normalized to cell viability which was determined using a Non-Radioactive Cell-Proliferation Assay Kit (Promega).

Quantitative Real-Time RT–PCR Analysis

Total RNA was isolated with TRIzol (Invitrogen) and subsequently subjected to cleanup using an RNase-Free DNase Set and RNeasy Mini kit (Qiagen). Quantitative real-time RT-PCR was performed as described previously (42). The primers (sequences are shown in Table S1, Supplementary Materials) were designed using Primer Express 4 (Applied Biosystems, Foster City, CA) and synthesized by MWG-BIOTECH Inc. (High Point, NC). Real-time fluorescence detection was performed using an ABI PRISM 7900 HT Fast Real-time PCR System (Applied Biosystems).

Western Blot Analysis

Isolation of cell fractions and western blotting were performed as detailed previously (41,43). The antibody for Nrf2 (sc-13032; 1:500) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies for β-ACTIN (A1978; 1:2000), LAMIN A (L1293; 1:2,500) and α-TUBULIN (T5168; 1:2,000) were purchased from Sigma.

Cell Viability

A minimum of five replicates of 10,000 cells/well were plated in 96-well plates and allowed to adhere to the plate for 24 h, after which the medium was removed and replaced with fresh medium containing vehicle or SFN. Cells viability was determined using the CellTiter Non-Radioactive Cell-Proliferation Assay Kit with MTS [3-(4,5-dimethylthiazol-2-yl)-2,5-diphen yltetrazolium bromide] (Promega) as described previously (44). Measurements are expressed as a percentage of untreated control cells.

Statistical Analyses

All statistical analyses were performed 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. The data are expressed as mean ± SD. For comparisons among groups, a one-way or two-way ANOVA with Bonferroni post hoc testing was performed.

Model Formulation

The mathematical model is primarily based on the molecular circuit schematically illustrated in Fig. 1. In the model, ROS are treated collectively as a single state variable, and antioxidant genes/enzymes also are treated as a single state variable named as AC, which represents the overall cellular Antioxidant Capacity. Because the role of sensor molecule Keap1 is to promote Nrf2 degradation, for simplicity, it is reasonable to omit Keap1 and assume that ROS and SFN directly inhibit the degradation process of Nrf2. Nrf2 then transcriptionally upregulates AC and AC is responsible for increasing the clearance of ROS. The following four ordinary differential equations describe the redox control circuit, its perturbation by SFN, as well as ROS- and glucose-stimulated insulin secretion. The state variables ROS, Nrf2, AC and insulin have arbitrary unit. The model was parameterized such that the steady-state levels of ROS, Nrf2, and AC are unity at basal conditions where SFN=0 and glucose=3 mM. The amount of insulin secreted in 30 min at the above condition was also parameterized to unity. All parameter values are listed in Table S2 (Supplementary

Materials). The model was constructed in Berkeley Madonna (University of California, Berleley, CA) and solved with the Rosenbrock stiff solver.

$$\frac{dROS}{dt} = k_0 + k_1 \cdot SFN + k_2 \cdot (Glu - 3) - k_3 \cdot AC \cdot ROS - k_4 \cdot Scavenger \cdot ROS \tag{1}$$

$$\frac{dNrf2}{dt} = k_5 - k_6 \cdot \frac{Nrf2}{1 + \frac{ROS}{J_1} + \frac{SFN}{J_2}} - k_7 \cdot Nrf2 \tag{2}$$

$$\frac{dAC}{dt} = k_8 + k_9 \cdot \frac{Nrf2^n}{J_3^n + Nrf2^n} - k_{10} \cdot AC \tag{3}$$

$$\frac{dInsulin}{dt} = k_{11} + k_{12} \cdot \frac{ROS}{J_4 + ROS} \cdot \frac{Glu}{J_5 + Glu} \tag{4}$$

RESULTS

Cellular redox homeostatic control is a dynamical process involving multiple transcriptional controls and antioxidant genes. Despite the complexity, its qualitative behavior as a dynamical control system can be rationalized by a general circuit structure involving both feedback and feedforward regulations (35). By formulating a simple mathematical model for this circuit, as illustrated in Fig. 1, we analyzed the dynamical and steady-state changes in ROS and antioxidant levels induced by Nrf2-activating antioxidant SFN. Qualitative predictions made with the model on basal and glucose-stimulated insulin secretion were then validated in cultured β-cells and/or mouse islets.

Adaptive Response of Simulated Redox Homeostatic Control Circuit

The simulated dynamical responses of ROS, Nrf2 and AC to perturbations by various concentrations of SFN follow a typical adaptive response pattern (Fig. 2a-c). Upon SFN challenge, the ROS level spikes immediately and gradually decreases over time till settling to a new steady state slightly above the baseline (Fig. 2a). This adaptive change in ROS occurs because Nrf2 is first activated by ROS through the feedback loop and by SFN through the feedforward loop (Fig. 2b), then it upregulates AC (Fig. 2c) to remove ROS until reaching a lower steady state.
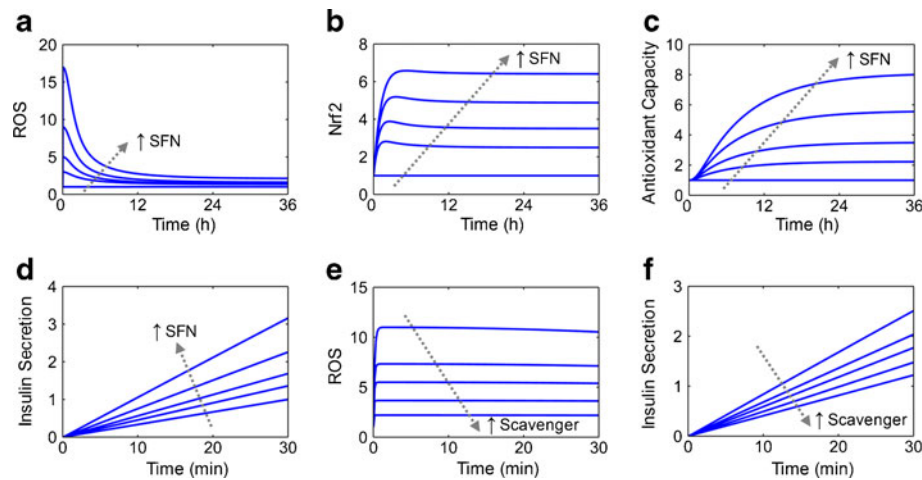
Fig. 2 Simulated dynamical responses to SFN. (**a**-**d**) Simulated time courses of ROS, Nrf2, AC and basal insulin secretion in response to continuous exposure to SFN at various concentrations (0, 4, 8, 16 and 32 μM). Except for insulin secretion, shown are fold increases over baseline levels. For insulin secretion, shown are the amount accumulated in the first 30 min of SFN treatment normalized to the accumulated amount for SFN=0. (**e** and **f**) Simulated effects of increasing ROS-scavenging activity on transient ROS and basal insulin secretion stimulated by SFN. Shown are simulations with scavenger at level 0, 0.5, 1, 2, 4 in response to 20 μM SFN.

Effect of Acute SFN Treatment on Basal Insulin Secretion

Because ROS can stimulate insulin secretion (18–22), the transient increase in ROS immediately following the onset of SFN treatment (Fig. 2a) is expected to acutely boost basal insulin secretion. The model showed that in the presence of low glucose (3 mM), secreted insulin accumulates to higher levels following SFN stimulation in a concentration-dependent manner (Fig. 2d). This acute, stimulatory effect of SFN was first validated in INS-1(832/13) cells, a cell line widely used to evaluate β-cell function *in vitro* (45). As shown in Fig. 3a, exposing the cells to SFN for 30 min concentration-dependently stimulated insulin secretion while glucose was kept at the basal level (3 mM). Other oxidative secretagogues, including hydrogen peroxide (H_2O_2), DEM and GO, produced similar stimulatory effects. DEM stimulates insulin secretion by depleting sulfhydryl to enhance ROS accumulation (21), whereas GO oxidizes glucose to generate H_2O_2. The stimulatory effect of acute SFN treatment also occurred with isolated mouse islets, where a modest but statistically significant increase in basal insulin secretion was observed (Fig. 3b). As expected, H_2O_2 also potently stimulated insulin release from the islets. As positive control, high-level glucose and KCl both readily stimulated insulin secretion in INS-1(832/13) cells and isolated mouse islets (Fig. 3a and b).

To determine whether the stimulation of insulin secretion by SFN involves ROS as a signaling intermediate, intracellular ROS levels in INS-1(832/13) cells were determined. As shown in Fig. 3c, 30-min SFN exposure concentration-dependently increased intracellular peroxide levels as measured by using CM-H₂DCFDA. To determine whether the observed increase in ROS by SFN was indeed involved in

stimulating insulin secretion, we examined the effects of SFN in the presence of ROS-scavenging agents. Both NAC and GSH-EE blocked SFN-stimulated insulin secretion from INS-1(832/13) cells (Fig. 3d). The mathematical model was also able to recapitulate this phenomenon, showing that increasing the rate of ROS scavenging dampened the transient increase in ROS stimulated by SFN (Fig. 2e) and consequently insulin secretion (Fig. 2f). Taken together, these results demonstrated that acute SFN treatment stimulates basal insulin secretion in β-cells, which is mediated, at least in part, by SFN-generated ROS.

Effects of Chronic SFN Treatment on Insulin Secretion

While the acute, stimulatory effect of SFN on basal insulin secretion can be readily explained by the initial transient ROS accumulation, the consequence of prolonged exposure to SFN is not as straightforward. As shown in Fig. 2a-c, cells persistently exposed to SFN are expected to have adapted and reached a new steady state, where Nrf2 and AC are markedly elevated but with only slightly increased ROS levels. This adapted state is likely to exert differential effects on basal insulin secretion *vs.* GSIS. The slightly increased ROS at the adapted state would only marginally boost basal insulin secretion, according to model simulations (Fig. 4a). This prediction was confirmed with INS-1(832/13) cells, which showed that continuous treatment with SFN at non-cytotoxic concentrations for 24 h caused only a fractional increase in basal insulin secretion under 3 mM glucose (Fig. 4e).

In the context of ROS functioning as signaling molecules, the effect of prolonged SFN exposure on GSIS depends on the direction in which glucose-stimulated ROS are
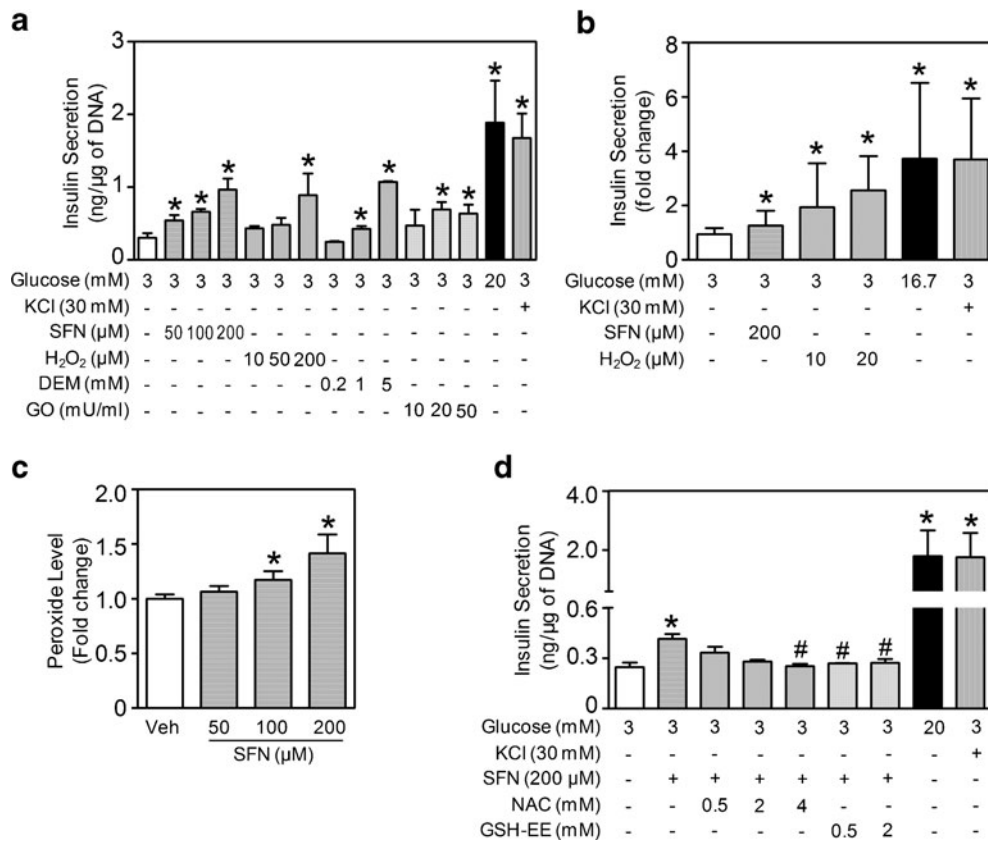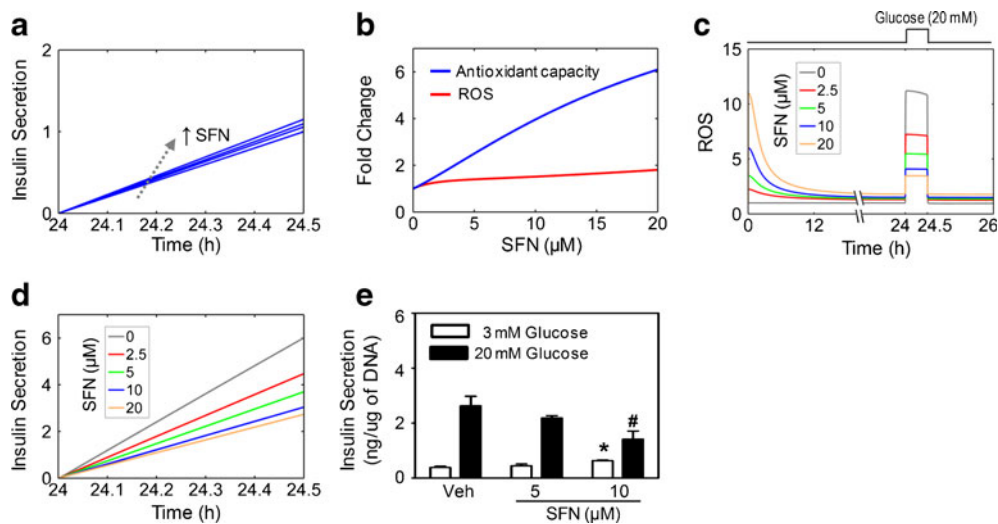
Fig. 3 Acute effect of SFN on basal insulin secretion and involvement of ROS. **a** Insulin secretion by INS-1(832/13) cells treated with SFN or other oxidative agents (H_2O_2, DEM and GO) at various indicated concentrations for 30 min under 3 mM glucose condition. High glucose (20 mM) and KCl were used as positive controls. Secreted insulin was normalized by DNA content. (**b**) Insulin secretion by isolated mouse islets treated with SFN or H_2O_2 at indicated concentrations for 30 min under 3 mM glucose condition. High glucose (16.7 mM) and KCl were used as positive controls. Secreted insulin was normalized by islet number. For panels (**a**) and (**b**), $n = 3$–6 independent experiments. *, $p < 0.05$ vs. 3 mM glucose alone. (**c**) SFN-stimulated intracellular peroxide production. INS-1(832/13) cells were challenged with SFN at various indicated concentrations for 30 min under 3 mM glucose condition. Veh, Vehicle (Kreb's buffer with 3 mM glucose). $n = 3$; *, $p < 0.05$ vs. Veh. (**d**) Pretreatment of INS-1(832/13) cells with ROS-scavenging antioxidants NAC or GSH-EE suppressed SFN-stimulated insulin secretion. Cells were pretreated with NAC or GSH-EE at indicated concentrations for 30 min followed by SFN stimulation for additional 30 min under 3 mM glucose condition. *, $p < 0.05$ vs. 3 mM glucose alone; #, $p < 0.05$ vs. 200 μM SFN at 3 mM glucose.

modulated at the adapted redox state. For the circuit in Fig. 1, the incoherent feedforward loop and potential ultrasensitive signaling motifs embedded in the feedback loop can amplify the induction of antioxidant enzymes by SFN, keeping the ROS level in check (35,39). The mathematical model recapitulated the final adapted state, which is characterized by a marginal increase in ROS but a higher fold increase in AC for a range of SFN concentrations (Fig. 4b), a state consistent with the antioxidant role of SFN. The small increase in basal ROS is expected to have little impact on glucose-stimulated ROS signal. In contrast, the large fold induction of AC is likely to markedly attenuate glucose-stimulated ROS signal. The model presaged that with prolonged exposure to SFN, the transient ROS signal stimulated by 20 mM glucose can be significantly attenuated, and the higher the SFN concentration, the smaller the glucose-stimulated ROS signal (Fig. 4c). Correspondingly, GSIS was predicted to be suppressed by SFN in a concentration-dependent manner (Fig. 4d).

We sought to verify the model predictions with INS-1(832/13) cells. Although activation of Nrf2-mediated antioxidant response by SFN has been reported in many cell types, including pancreatic β-cells (28), it is unknown whether the response indeed occurs in INS-1(832/13) cells. As shown in Fig. 5a, 6-h SFN treatment markedly increased cellular Nrf2 protein levels in a concentration-dependent fashion. As with other well-known Nrf2 activators, such as arsenite and tert-butylhydroquinone (tBHQ), SFN resulted in a substantial nuclear accumulation of Nrf2 protein (Fig. 5b). In addition, INS-1(832/13) cells stably transfected with an ARE-luciferase reporter showed concentration-dependent induction of luciferase activity following SFN treatment (Fig. 5c). Lastly, expression of antioxidant genes, such as sulfiredoxin 1 (*Srxn1*), heme oxygenase 1 (*Hmox1*), γ-glutamate cysteine ligase catalytic subunit (*Gclc*) and NAD(P)H: quinone oxidoreductase 1 (*Nqo1*) were all significantly induced by SFN (Fig. 5d).

Fig. 4 Chronic effects of SFN on basal insulin secretion and GSIS. (**a**) Simulated basal insulin secretion in the last 30 min of continuous exposure to SFN at various concentrations (0, 2, 5, 10, and 20 μM) for 24.5 h (**b**) Simulated dose responses of antioxidant capacity and ROS following continuous exposure to SFN for 24 h. (**c**) Simulated attenuation of glucose-stimulated ROS signal by SFN. SFN is present continuously for 26 h and glucose is switched from 3 to 20 mM at 24 h and back to 3 mM at 24.5 h as indicated by the square wave on top. (**d**) Simulated GSIS during the 30 min (24–24.5 h) when glucose is at 20 mM as in (**c**). (**e**) Experimentally measured basal insulin secretion (*white bar*) or GSIS (*black bar*) in INS-1(832/13) cells following 24-h SFN exposure at concentrations indicated. Secreted insulin was normalized by DNA content. $n = 3$–6 independent experiments; *, $p < 0.05$ *vs*. Veh with 3 mM glucose; #, $p < 0.05$ *vs*. Veh with 20 mM glucose.

With the knowledge that SFN indeed activates Nrf2 and the adaptive antioxidant response in INS-1(832/13) cells, we then sought to test the model's prediction on the effect of chronic SFN treatment on GSIS. For INS-1(832/13) cells that were continuously incubated with SFN at low, non-cytotoxic concentrations for 24 h, insulin secretion stimulated by 20 mM glucose was significantly attenuated in comparison to control cells, and a higher SFN concentration resulted in a more significant suppression of GSIS (Fig. 4e). This result is highly consistent with the model prediction shown in Fig. 4d. The attenuation of GSIS is not likely to result from cytotoxic damage to the insulin secretory apparatus by SFN because the
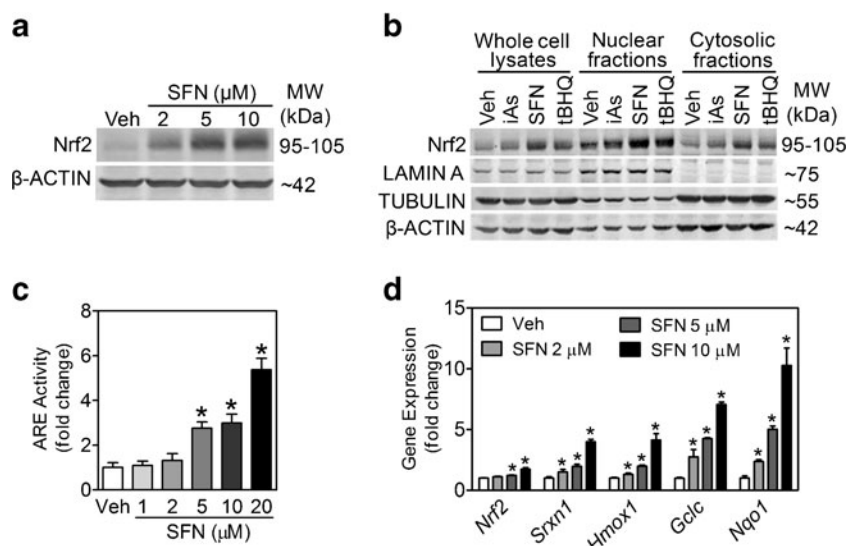
Fig. 5 Activation of Nrf2 and antioxidant response by SFN in INS-1(832/13) cells. (**a**) SFN concentration-dependently increased Nrf2 protein levels in INS-1(832/13) cells. Cells were treated with SFN for 6 h, and whole-cell lysates were used for immunoblotting. (**b**) SFN increased Nrf2 protein accumulation in nuclear fractions. Whole cell lysates, nuclear fractions and cytosolic fractions were collected after treatment with Veh (medium) and the indicated agents for 6 h. iAs, arsenite (5 μM); SFN (10 μM); tBHQ, tert-butylhydroquinone (50 μM). LAMIN A, TUBULIN and β-ACTIN were used as loading controls for nuclear fractions, cytosolic fractions and whole cell lysates, respectively. (**c**) SFN concentration-dependently induced ARE-luciferase activity. Cells were treated with SFN for 9 h. $n = 4$; *, $p < 0.05$ *vs*. Veh (medium). (**d**) Transcripts of Nrf2 and ARE-dependent genes measured by real-time RT-PCR. Cells were treated with SFN for 6 h. $n = 2$–5 independent experiments; *, $p < 0.05$ *vs*. Veh.

SFN concentrations used were non-cytotoxic (Fig. 6a), and more over, basal insulin secretion was not compromised, but rather slightly increased at the same concentrations (Fig. 4e). To further prove that SFN at low concentrations is indeed antioxidant and cytoprotective, we pretreated INS-1(832/13) cells with 2 µM SFN for 24 h and subsequently exposed them to various concentrations of H_2O_2 for an additional 6 h. Pretreatment with SFN led to a significantly increased resistance to cellular toxicity by H_2O_2, as evidenced by the marked right-ward shift in the cell viability curve (Fig. 6b). In accordance with the indirect antioxidant role of SFN, the cytoprotective effects likely resulted from the induction of endogenous antioxidants at low concentrations of SFN (Fig. 5d).
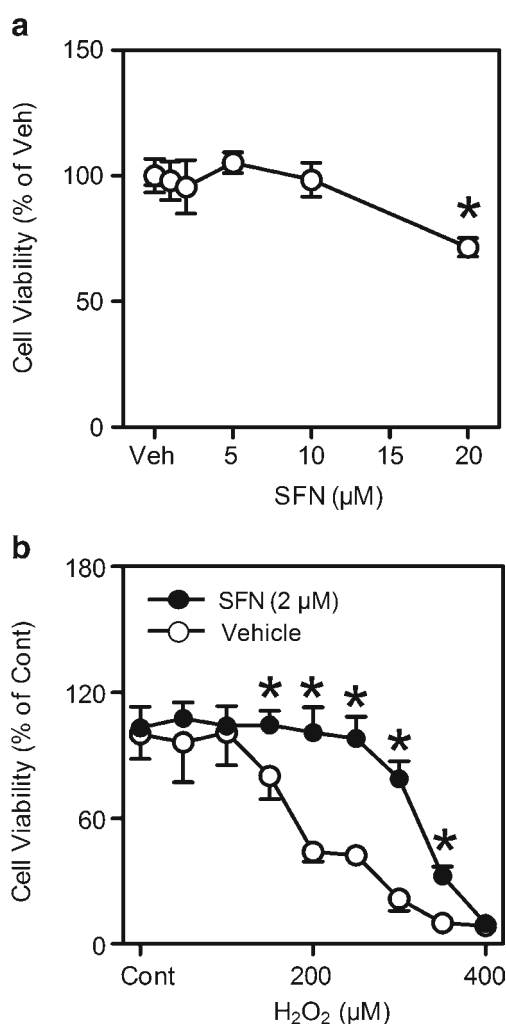
Fig. 6 Low, non-cytotoxic concentrations of SFN protected cells from H_2O_2-induced cytotoxicity. (**a**) Cytotoxicity of SFN in INS-1(832/13) cells. Cell viability was measured with the MTT assay following SFN treatment for 24 h. (**b**) INS-1(832/13) cells were pretreated with 2 µM SFN or Vehicle (medium) for 24 h, followed by H_2O_2 exposure for 6 h and measurement of cell viability. $n = 4$; *, $p < 0.05$ vs. Vehicle with the same-concentration H_2O_2 treatment.

DISCUSSION

Oxidative damage of pancreatic β-cells by excessive ROS has been regarded as a key pathogenic factor for β-cell failure (6). Recent advances in β-cell study have revealed a physiological role for ROS, in particular H_2O_2, in mediating GSIS (18–22). This paradox makes evaluating the effects of redox-altering compounds on insulin secretion a challenging task. Further complicating the matter is the fact that many redox-altering compounds can be either pro- or antioxidants, depending on the concentrations and duration applied, as the cellular redox balance responds dynamically to the chemical perturbations. In the present study, we dissected the effects of SFN, a typical plant-derived Nrf2-activating antioxidant, on both basal and glucose-stimulated insulin secretion. We utilized a mathematical model to capture the dynamical changes of ROS and antioxidant capacity in the process of adaptive antioxidant induction and redox homeostatic control. The integrated study reveals that an acute challenge of INS-1(832/13) cells or cultured mouse islets with SFN triggers insulin secretion, which is mediated by SFN-stimulated transient ROS accumulation. Conversely, due to activation of Nrf2 and induction of downstream antioxidant enzymes, prolonged SFN exposure attenuates glucose-stimulated ROS and GSIS, albeit protecting the cells from future oxidative insults. The findings suggest that as an Nrf2-activator, SFN may unnecessarily stimulate basal insulin release during fasting when insulin is not needed, but inhibit postprandial GSIS when insulin is needed for maintaining blood glucose levels.

Growing evidence indicates that ROS can function as second messengers that serve an intracellular signaling role in a variety of organs or tissues involved in glucose metabolism (46,47). In addition to pancreatic β-cells (21,22), H_2O_2 as a second messenger is involved in insulin signaling in the liver, adipose tissue and skeletal muscle where glucose uptake takes place, and in glucose sensing in the hypothalamus where feeding behavior is regulated (23,48,49). Given the physiological role of ROS in these multiple processes regulating glucose catabolism, it is reasonable to argue that unduly boosting intracellular antioxidant levels may have pathogenic consequences with the development of diabetes. In keeping with this notion, it was demonstrated that mice overexpressing H_2O_2-metabolizing enzyme glutathione peroxidase or catalase exhibit decreased insulin sensitivity, impaired β-cell function, and accelerated development of obesity and diabetes (50,51).

The conventional view of ROS as a pathogenic factor has led to the advocacy of antioxidant supplementation to promote general health, including preventing and treating diabetes. Although certain clinical studies with antioxidant supplements showed health benefits in a limited number of settings such as cancer prevention (52), substantial evidence

indicates antioxidant therapy is not as effective as expected for many disease endpoints including diabetes (8–17). While the ineffectiveness may be attributed to many clinical factors, by cancelling out the benefit of cytoprotection provided by exogenous antioxidants, the inhibitory action of antioxidants on GSIS, as observed here *in vitro* with SFN, as well as the potential inhibition by antioxidants of insulin action in the adipose tissue and liver, and of glucose-sensing in the hypothalamus, could all contribute to the observed ineffectiveness,

While the inhibitory effect of SFN on GSIS observed here *in vitro* is attributed to enhanced antioxidant capacity, SFN may also perturb other signaling pathways that are involved in GSIS. One candidate pathway is NF-κB, whose transcriptional activity appears to be necessary for GSIS, as attenuation of NF-kB activation disrupted the expression of genes involved in glucose uptake, oxidative metabolism, and insulin exocytosis (53–55). A number of studies have demonstrated that SFN and other isothiocyanate derivatives can inhibit the transcriptional activation of NF-κB (56,57). Therefore it is likely that inhibition of GSIS by SFN may also be mediated in part by suppression of NF-κB. In addition, the NF-κB-mediated transcriptional response plays a crucial role in regulating β-cell proliferation and apoptosis in response to cytokines involved in inflammation, with the outcome dependent on the magnitude and duration of NF-κB signaling (58–61). The anti-inflammatory function of SFN through suppressing NF-κB suggests that SFN may have an effect on β-cell fate in islets *in vivo*, by which insulin secretion can be altered.

GSIS is regulated by the rate of glucose metabolism within β-cells. Increased glucose leads to an increase in the glycolytic flux and an acceleration of mitochondrial NADH production. Oxidation of NADH increases ATP synthesis, decreases ADP concentration and thus increases ATP/ADP ratio. These processes are critically important for insulin secretion. Our previous studies demonstrated that ROS alone cannot increase insulin secretion without ATP (21), which suggests that the effects of SFN on insulin secretion may eventually rely on changes in the ATP/ADP ratio. In addition, shuttling of glycolysis-derived NADH into mitochondria has been shown to play important role in mediating GSIS (62). It appears that SFN may increase the NAD(P)H/NAD(P) ratios, as shown in other cell types (63,64). However, it remains to be seen whether the effects of SFN, acutely or chronically, on insulin secretion can be mediated in part through perturbing the ratios of the redox pairs in β-cells.

Given the common antioxidant mechanism for many plant-derived Nrf2-activating compounds, such as curcumin, catechin, resveratrol, and oleanolic acid, it is tempting to surmise that these compounds may dysregulate insulin secretion in a similar manner as SFN, especially if their primary action is on the cellular redox system. Numerous studies using cultured β-cells or isolated animal islets seem to support that these compounds are largely stimulatory to basal insulin secretion (65–69). Yet their effects on GSIS are much less consistent; both augmentation and suppression of GSIS by these compounds have been observed *in vitro* (65,67,69–73). While time and compound concentration used in these studies are variables that may explain some of these discrepancies, another important possibility is that many of these compounds can regulate processes other than Nrf2 activation. For instance, the inhibition of GSIS by resveratrol has been attributed to multiple processes that resveratrol may affect, including blockade of voltage-gated Ca^{2+} channels and shifting of glucose metabolism from mitochondrial oxidation to anaerobic catabolism (71,74). Therefore the final outcome of Nrf2-activating compounds on GSIS is likely the result of combined effects of multi-pathway modulation by these compounds. While much of information is available for the effects of Nrf2 activators on insulin secretion, the consequence of Nrf2 inhibition has not been explored. Recently it was discovered that brusatol, extracted from Brucea javanica (L) Merr., can inhibit Nrf2 by enhancing its ubiquitination and proteasomal degradation (75). By increasing the degradation rate constants of Nrf2 to mimic the effect of brusatol, our model simulation showed that basal Nrf2 level and antioxidant capacity are lowered and basal ROS level is increased. As a result, the model predicted that in the presence of brusatol, basal insulin secretion is elevated, but contrary to the effect of SFN, GSIS is also augmented (simulation results not shown). Such predictions remain to be validated by further experimental studies.

As demonstrated in the present study, SFN can protect β-cells from the cytotoxicity induced by exogenous oxidative stressors such as H_2O_2. However, the surviving β-cells are not necessarily functioning equally well as non-stressed cells. The induction of endogenous antioxidants in those stressed but surviving β-cells can blunt GSIS. Although effective as chemoprevention agents, the long-term health consequence of Nrf2-activating compounds in non-cancer endpoints is still unclear. The finding that prolonged SFN treatment results in attenuated GSIS in β-cells raised a reasonable concern over the application of SFN and other Nrf2-activating antioxidants in chemoprevention since insulin deficiency may become a tangible side effect. Altering the cellular redox state through antioxidant supplementation may do both good—and harm, through potentially inhibiting physiological H_2O_2—thus producing no overall health benefit. It is likely difficult to strike the right balance by optimizing the dose to achieve overall beneficial results. The present study suggests that the timing of antioxidant supplementation might be a factor to be considered in order to minimize the potential side effect on insulin secretion. For instance, by taking Nrf2-activating antioxidants at meal time, it may (a) help stimulate insulin secretion given their

initial ROS-generating effect and (b) minimize inhibition of GSIS because it takes several hours for endogenous antioxidants to be induced in β-cells, by which time, blood glucose would have receded and insulin is no longer needed. Nevertheless, this postulated effect of timing clearly needs further experimental investigations in whole animals.

Although SFN is generally regarded as an antioxidant because it induces endogenous antioxidant enzymes, it is quite intriguing to note that arsenite, an environmental oxidative chemical, exhibits similar divergent effects on insulin secretion. We have demonstrated previously with INS-1(832/13) cells and isolated mouse islets that arsenite acutely stimulated insulin secretion under basal glucose conditions, and prolonged exposure of these cells to non-cytotoxic arsenite markedly blunted GSIS (21,40). The inhibitory effect on insulin secretion is due to the induction of endogenous antioxidant enzymes by arsenite, which blocks glucose-stimulated signaling ROS. We have thus proposed that adaptive induction of endogenous antioxidant enzymes, rather than oxidative damage, is the primary pathogenic mechanism by which low-level environmental oxidative stressors impair β-cell function. In light of this premise, combating environmental oxidative stress with antioxidant supplements such as SFN, which also induces endogenous antioxidant genes, can be futile if not worsening the GSIS response. Nevertheless, the cytoprotection provided by SFN and other Nrf2-activating compounds—achieved by the induction of phase II enzymes and antioxidants to promote detoxification of oxidative chemicals and their reactive metabolites—cannot be dismissed. Antioxidant compounds that can directly scavenge oxidative chemicals in extracellular compartments without interfering with intracellular signaling ROS are likely to be more effective therapeutically with minimal side effect and therefore should be developed.

Interpreting and predicting experimental results in biological systems have increasingly relied on simple but adequate mathematical models of the underlying molecular circuits. The antioxidant response model formulated here followed the principle of parsimony by excluding details of regulatory interactions and metabolic reactions catalyzed by a variety of antioxidant enzymes. Yet the nonlinearity and dynamics captured by the simplified model, which are key to our general understanding of the system, are unlikely to be qualitatively different from more complicated redox control circuits that share the same overall feedback and feedforward structure (35). Importantly, predictions made with this simple model were corroborated by experimental results.

In conclusion, this integrated study demonstrates that SFN has a paradoxical role in pancreatic β-cell function. First, SFN may stimulate basal insulin secretion through transient ROS production; Second, SFN activates Nrf2-mediated antioxidant response and protects β-cells from oxidative and electrophilic stress-induced cell damage. Third, prolonged SFN exposure may blunt ROS signaling and attenuate GSIS in pancreatic β-cells. To develop therapeutic approaches using SFN and other potent Nrf2 activators, further investigations are needed.

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