Free Radical Research, February 2007; 41(2):242–250

informa
healthcare

Upregulation of endogenous glutathione system by 3*H*-1,2-dithiole-3-thione in pancreatic RINm5F beta-cells as a novel strategy for protecting against oxidative beta-cell injury

HONG ZHU[1], LI ZHANG[1], MICHAEL A. TRUSH[2], & YUNBO LI[1]

[1]*Department of Internal Medicine and Davis Heart & Lung Research Institute, The Ohio State University College of Medicine and Public Health, 473 West 12th Avenue, Columbus, OH 43210, USA, and* [2]*Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA*

Accepted by Dr E. Niki

(Received 25 July 2006; in revised form 30 August 2006)

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

Abstract

This study was undertaken to investigate the inducibility of glutathione (GSH), glutathione reductase (GR) and glutathione peroxidase (GPx) by 3*H*-1,2-dithiole-3-thione (D3T) in beta-cells, and the resultant cytoprotection against oxidant injury. Incubation of the insulin-secreting RINm5F cells with D3T led to significant induction of GSH, GR and GPx. D3T-mediated induction of GSH was abolished by buthionine sulfoximine (BSO), suggesting a critical involvement of γ-glutamylcysteine ligase (γGCL). Consistently, incubation of RINm5F cells with D3T resulted in increased expression of γGCL protein and mRNA. Pretreatment of RINm5F cells with D3T provided remarkable protection against oxidant-elicited cytotoxicity. On the other hand, depletion of cellular GSH by BSO sensitized RINm5F cells to oxidant injury. Furthermore, cotreatment of RINm5F cells with BSO to reverse D3T-mediated GSH induction abolished the cytoprotective effects of D3T on oxidant injury. Taken together, this study demonstrates that upregulation of glutathione system by D3T is effective for protecting against oxidative beta-cell injury.

Keywords: *3*H*-1,2-dithiole-3-thione, glutathione system, RINm5F beta-cells, reactive oxygen species, reactive nitrogen species, cytotoxicity*

Abbreviations: *BSO, buthionine sulfoximine; D3T, 3*H*-1,2-dithiole-3-thione; FBS, fetal bovine serum; **γGCL, γ-**glutamylcysteine ligase; **γ**GCL-C, **γ**-glutamylcysteine ligase catalytic subunit; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, rduced glutathione; GSSG, oxidized form of glutathione; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; PBS, phosphate buffered saline; ROS, reactive oxygen species; RNS, reactive nitrogen species; SIN-1, 3-morpholinosydnonimine*

Introduction

Diabetes mellitus has become an epidemic, affecting more than 170 million individuals worldwide [1]. Beta-cell injury/dysfunction is a critical feature of the pathophysiology of both type 1 and type 2 diabetes [2,3]. Although the mechanisms underlying pancreatic beta-cell injury/dysfunction are complex, accumulating evidence supports a causal involvement for oxidative stress in the pathophysiological processes, leading to beta-cell injury [4,5]. In this context, it has been reported that pancreatic beta-cells are relatively deficient in antioxidant defenses [6,7], which may render these cells particular vulnerability to oxidative injury.

Correspondence: Y. Li, Edward Via Virginia College of Osteopathic Medicine, VTCRC, 2265 Kraft Drive, Blackbwg, VA 24060, USA. Tel: 1 540 231 1465. E-mail: yli@vcom.vt.edu

ISSN 1071-5762 print/ISSN 1029-2470 online © 2007 Informa UK Ltd.
DOI: 10.1080/10715760601009586

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

It has been suggested that augmented formation of reactive oxygen species (ROS) in pancreatic islets can result from hyperglycemia [4,5]. On the other hand, inflammatory responses, seen particularly in type I diabetes, may also lead to increased production of ROS as well as reactive nitrogen species (RNS) in the pancreatic islets, which may further exacerbate beta-cell degeneration [8,9]. The appreciation of ROS and RNS as critical players in pancreatic beta-cell injury has led to extensive studies on the protective effects of antioxidants in diabetes in both animal models and human clinical trials [10]. In this regard, although not being practical in humans, transgenic overexpression of antioxidant proteins, such as thioredoxin, in pancreatic islets has been reported to protect mice from experimental diabetes [11]. Similarly, overexpression of antioxidant enzymes, including glutathione peroxidase (GPx) via genetic approach has been found to protect against oxidative injury in cultured pancreatic beta-cells [12,13]. More recently, Tran et al. has demonstrated that overexpression of γ-glutamylcysteine ligase (γGCL), a key enzyme in GSH biosynthesis, protects pancreatic islets from oxidative stress [14]. These studies point to a critical role for the glutathione system in protecting against oxidative beta cell injury. Indeed, due to the unusually low activities of SOD, catalase, and GPx in pancreatic beta-cells, GSH has been suggested to be a major cellular defense against oxidative stress in pancreatic islets [15].

In addition to the genetic approach, elevation of cellular glutathione system can be achieved via the use of pharmacological agents. For instance, the unique chemoprotectant, D3T has been shown to upregulate endogenous glutathione system in several types of tissues/cells [16–18]. However, whether the endogenous glutathione system, including GSH, glutathione reductase (GR) and GPx, in pancreatic beta-cells can also be induced by D3T had not been previously reported. In this study, using rat insulin-secreting pancreatic RINm5F cells, a commonly used *in vitro* model for studies on beta cell physiology and pathophysiology [19–21], we have determined the inducibility of endogenous GSH, GR, and GPx by D3T, and the cytoprotective effects of the D3T-upregulated glutathione system on oxidative beta-cell injury. The results of this study demonstrated that GSH, GR and GPx could all be induced by D3T and that the D3T-augemented GSH biosynthesis appeared to be the predominant mechanism underlying D3T-mediated cytoprotection against ROS/RNS-elicited beta-cell injury.

Materials and methods

Materials

D3T with a purity of 99.8% was generously provided by Dr Mary Tanga at SRI International (Menlo Park, CA, USA) and Dr Linda Brady at National Institute of Mental Health (Bethesda, MD, USA). RPMI 1640 medium, penicillin, streptomycin, and fetal bovine serum (FBS) were from Gibco-Invitrogen (Carlsbad, CA, USA). All other chemicals and reagents were from Sigma Chemicals (St Louis, MO, USA).

Cell culture

Rat pancreatic RINm5F beta-cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. The cells were fed every 3–4 days, and subcultured once they reached 80–90% confluence.

Preparation of cell extract

The cells were collected and resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at 13,000*g* for 10 min at 4°C. The resulting supernatants were collected and the protein concentrations were quantified with Bio-Rad protein assay dye (Hercules, CA, USA) using bovine serum albumin as the standard. The samples were kept on ice for measurement of GSH content and activities of GR and GPx within 2–3 h.

Assay for GSH content

The level of cellular GSH was measured according to the fluorometric method, which is specific for measurement of GSH at a pH of 8.0 [22]. Briefly, 10 μl of the sample was incubated with 12.5 μl of 25% metaphosphoric acid, and 37 μl of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0 at 4°C for 10 min. The samples were centrifuged at 13,000*g* for 5 min at 4°C. The resulting supernatant (10 μl) was incubated with 0.1 ml of *o*-phthalaldehyde solution (0.1% in methanol) and 1.89 ml of the above phosphate buffer for 15 min at room temperature. Fluorescence intensity was then measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. GSH content was calculated using a concurrently run GSH (Sigma) standard curve, and expressed as nmol of GSH per mg of cellular protein.

Assay for GR activity

The method described before [22] was followed to measure the activity of cellular GR in a final reaction volume of 0.6 ml. Briefly, to an assay cuvette containing 0.46 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA, 20 μl of sample and 60 μl of 20 mM oxidized form of glutathione (GSSG) were

RIGHTSLINK

added. The cuvettes were pre-warmed at 37°C for 3 min. The reaction was started by adding 60 μl of 1.5 mM NADPH (prepared in 0.1% $NaHCO_3$). The subsequent consumption of NADPH was monitored at 340 nm, 37°C for 5 min. GR activity was calculated using the extinction coefficient of $6.22\,mM^{-1}\,cm^{-1}$, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

Assay for GPx activity

Cellular GPx activity was measured as described before [22] in a final reaction volume of 0.6 ml. In brief, to an assay cuvette containing 0.34 ml of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA and 2 mM sodium azide, 20 μl of sample, 60 μl of 10 mM GSH, 60 μl of glutathione reductase (2.4 U/ml, Sigma) and 60 μl of 1.5 mM NADPH (prepared in 0.1% $NaHCO_3$) were added. The cuvette was incubated at 37°C for 3 min. After addition of 60 μl of 2 mM H_2O_2, the rate of NADPH consumption was monitored at 340 nm, 37°C for 5 min. GPx activity was calculated using the extinction coefficient of $6.22\,mM^{-1}\,cm^{-1}$, and expressed as nmol of NADPH consumed per min per mg of cellular protein.

Immunoblot analysis

The procedures described before [23] were followed to detect γGCL protein expression by immunoblot analysis. Briefly, cells were lysed by sonication followed by centrifugation to yield the supernatant samples. Equal amounts of protein from each of the samples were resolved by SDS-PAGE on 10% gels, and transferred electrophoretically to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 5% non-fat dried milk in TTBS buffer at room temperature for 1.5 h. The membrane was then incubated with primary anti-γGCL (LabVision, Fermont, CA, USA) antibody overnight at 4°C, followed by incubation with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) at room temperature for another 1.5 h. The membrane was visualized using an enhanced chemiluminescence system (Amersham Biosciences) and blots were quantified by an Alpha Innotech Imaging system.

RT-PCR analysis of mRNA expression

Total RNA from RINm5F cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. cDNA synthesis and subsequent PCR reaction were performed using Superscript II One-Step system (Invitrogen) in a volume of 25 μl according to manufacturer's instruction. The cycling conditions for RT-PCR were the following: 50°C for 30 min (reverse transcription), 94°C for 2 min (pre-denaturation), followed by 25 cycles of PCR amplification process including denaturing at 94°C for 15 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s, and by 1 cycle of final extension at 72°C for 10 min. The PCR primers for γGCL catalytic subunit (γGCL-C) and β-actin were designed based on sequences in Genebank as described before [18]. PCR products were separated by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide and then analyzed under ultraviolet light using an Alpha Innotech Imaging system. In this study, a standard curve using 25–200 ng of total RNA was included to reliably detect changes in mRNA levels, as described previously [18].

Detection of cytotoxicity by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) reduction assay

For cytotoxicity studies, RINm5F cells were incubated with ROS or RNS in RPMI 1640 medium supplemented with 0.5% FBS in 24-well culture plates at 37°C for 24 h. After this incubation, the media were collected for measurement of LDH release as described below. Then, to each well, 0.5 ml MTT solution (0.1 mg MTT in RPMI 1640 medium supplemented with 0.5% FBS) was added. The plates were incubated at 37°C for another 2 h. After this 2-h incubation, media were removed and wells were rinsed once with PBS. To each well, 0.6 ml of mix of dimethyl sulfoxide, isopropanol and deionized water (1:4:5) was added at room temperature to solubilize the formazan crystals. The dissolved formazan was then transferred into semi-micro cuvettes, and the absorbance was measured at 570 nm [23].

Detection of cytotoxicity by lactate dehydrogenase (LDH) release assay

The method described before [23], was used with modifications to measure LDH activity directly in the media collected from cell cultures. Briefly, to an assay cuvette, 250 μl of culture medium, 190 μl of PBS and 30 μl of sodium pyruvate (5.5 mM prepared in PBS) were added. Then 30 μl of NADH (4 mM prepared in PBS) was added to start the reaction. The LDH-catalyzed NADH consumption was monitored at 340 nm, 25°C for 5 min. LDH activity was calculated using the extinction coefficient of $6.22\,mM^{-1}\,cm^{-1}$, and expressed as nmoles of NADH consumed per min per ml of culture medium.

Statistical analysis

All data are expressed as means ± SEM from at least 3 separate experiments. Differences between mean values of multiple groups were analyzed by one-way

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

RIGHTSLINK

analysis of variance (ANOVA) followed by Student–Newman–Keuls test. Differences between two groups were analyzed by Student *t*-test. Statistical significance was considered at $p < 0.05$.

Results

Induction of GSH, GR and GPx by D3T in RINm5F cells

GSH, GR, and GPx are key cytoprotective factors in the detoxification of both ROS and RNS in mammalian tissues/cells, including pancreatic islets [24,25]. We first determined the basal levels/activities of these antioxidants and their inducibility by D3T in RINm5F cells. RINm5F cells expressed constitutively a level of GSH similar to that in other types of cells, whereas the basal activity of GR in these cells was much higher than that in most other cell types (Figure 1(A),(B)). In contrast, the basal activity of GPx was extremely low (Figure 1(C)), which was in line with previous observations that pancreatic islets are relatively deficient in GPx [6,7]. Incubation of RINm5F cells with 10, 25 and 50 μM D3T for 24 h resulted in a marked induction of cellular GSH and GPx in a concentration-dependent manner (Figure 1(A),(C)), whereas the same D3T treatment only caused a small, but significant induction of cellular GR (Figure 1(B)). In data not shown, D3T alone at the above concentrations did not significantly affect the viability of RINm5F cells.

Induction of cellular GSH by D3T also exhibited a time-dependent fashion (Figure 2). A significant increase in GSH level was seen 3 h after treatment of RINm5F cells with 50 μM D3T; longer time of incubation with D3T led to further increases in cellular GSH content, which reached a maximal 3-fold induction at 24 and 48 h of D3T treatment (Figure 2). Since GSH has been proposed as a key cytoprotective factor in pancreatic beta-cells, we next investigated the mechanism(s) underlying D3T-mediated GSH elevation in RINm5F cells. To this end, we treated the cells with buthionine sulfoximine (BSO), a specific and potent inhibitor of γGCL, the key enzyme in GSH biosynthesis [26]. As shown in Figure 3(A), incubation of RINm5F cells with 25–100 μM BSO for 24 h caused a 75–80% decrease in cellular GSH content. BSO treatment alone did not cause any significant changes in cell viability (data not shown). Co-treatment of RINmF5 cells with 25 μM BSO led to a complete abolishment of cellular GSH elevation induced by 50 μM D3T (Figure 3(B)), suggesting a critical role for γGCL in D3T-mediated GSH induction.

To further investigate the involvement of γGCL in D3T-mediated GSH elevation, we examined the effects of D3T treatment on the protein and mRNA expression of γGCL. As shown in Figure 4(A), incubation of RINm5F cells with D3T resulted in a significantly increased expression of γGCL protein in a concentration-dependent fashion. Incubation of RINm5F cells with 50 μM D3T also led to significant time-dependent increases in the level of mRNA for γGCL-C (Figure 4(C)). The mRNA level for γGCL-C began to increase 3 h after D3T treatment; an overall 1.8–2.5-fold increase in the mRNA level was observed between 6 and 24 h (Figure 4(C)).

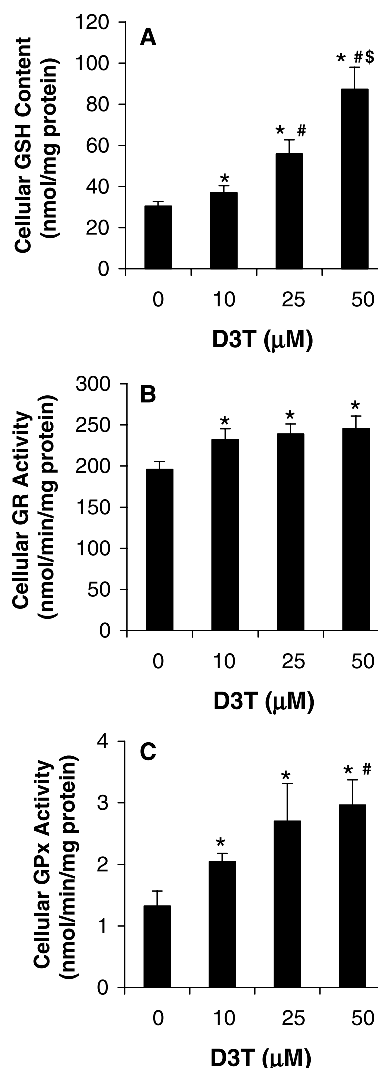

Figure 1. Induction of cellular GSH (A), GR (B) and GPx (C) by D3T in RINm5F cells. Cells were incubated with the indicated concentrations of D3T for 24 h, followed by measurement of GSH content and activities of GR and GPx. Values represent means ± SEM from 3–4 independent experiments. *, significantly different from control; #, significantly different from 10 μM D3T; $, significantly different from 25 μM D3T.

Protective effects of D3T pretreatment on ROS/RNS-elicited RINm5F cell injury

To examine if the D3T-elevated cellular defenses could lead to cytoprotection against oxidative injury,

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

RIGHTSLINK

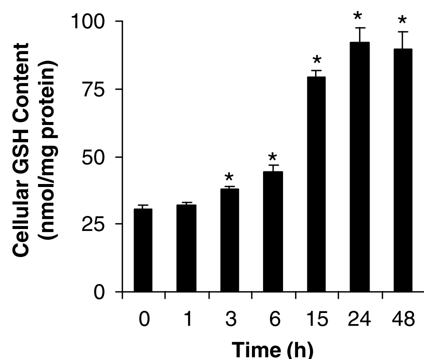

Figure 2. Time-dependent induction of cellular GSH by D3T in RINm5F cells. Cells were incubated with 50 μM D3T for the indicated time points, followed by measurement of GSH content. Values represent means ± SEM from four independent experiments. *, significantly different from 0 h.

RINm5F cells were pretreated with D3T and then exposed to H_2O_2 or the peroxynitrite generator, 3-morpholinosydnonimine (SIN-1). As shown in Figure 5, incubation of RINm5F cells with various concentrations of H_2O_2 (5–25 μM) or SIN-1 (25–125 μM) for 24 h led to significant decreases in cell viability, as detected by MTT reduction and LDH release assay. The decreases in cell viability were dependent on the concentrations of the ROS/RNS used. Pretreatment of RINm5F cells with 50 μM D3T for 24 h afforded a remarkable protection against the above ROS/RNS-induced cytotoxicity (Figure 5).

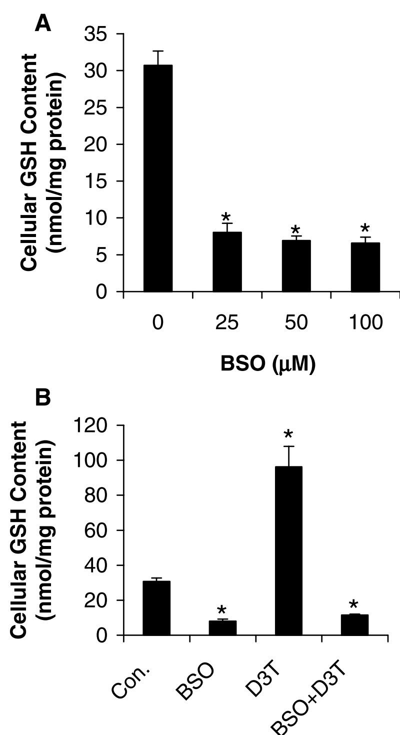

Figure 3. Cellular GSH depletion by BSO (A) and the effect of BSO cotreatment on D3T-mediated GSH elevation (B) in RINm5F cells. In A, cells were incubated with the indicated concentrations of BSO for 24 h, followed by measurement of cellular GSH content. In B, cells were incubated with 25 μM BSO, 50 μM D3T, or 25 μM BSO + 50 μM D3T for 24 h, followed by measurement of cellular GSH content. Values represent means ± SEM from three independent experiments. *, significantly different from 0 μM BSO (A) or control (B).

Potentiation of ROS/RNS-induced cytotoxicity by BSO in RINm5F cells

As shown in Figure 3, incubation of RINm5F cells with 25–100 μM BSO led to a dramatic 75–80% decrease in cellular GSH content without compromising cell viability. To determine if depletion of cellular GSH by BSO potentiated ROS/RNS-induced toxicity, RINm5F cells were pretreated with 25 μM BSO for 24 h, and then exposed to various concentrations of H_2O_2 or SIN-1 for another 24 h. As shown in Figure 6, pretreatment with BSO dramatically sensitized RINm5F cells to H_2O_2 or SIN-1-elicited cytotoxicity at all of the concentrations examined.

Effects of BSO cotreatment on D3T-mediated cytoprotection in RINm5F cells

To further investigate the causal involvement of GSH in D3T-mediated cytoprotection against ROS/RNS toxicity, RINm5F cells were pretreated with 50 μM D3T in the presence of 25 μM BSO followed by exposure to H_2O_2 or SIN-1. In this experiment, without BSO pretreatment, 25 μM H_2O_2 or 100 μM SIN-1 was the lowest concentration that induced an 80% decrease in cell viability as indicated by the MTT reduction assay (Figures 5 and 6), whereas 20 μM H_2O_2 or 75 μM SIN-1 was the lowest concentration that induced a similar 80% decrease in cell viability in the BSO-pretreated RINm5F cells (Figure 6). As shown in Figure 7(A),(B), in the absence of BSO cotreatment, the cytotoxicity induced by 25 μM H_2O_2 was markedly protected by D3T pretreatment. However, the protective effect of D3T on H_2O_2-induced cytotoxicity was remarkably reduced in BSO-pretreated cells. Similarly, the cytotoxicity elicited by 100 μM SIN-1 in the absence of BSO cotreatment was dramatically protected by D3T pretreatment, whereas in BSO-pretreated cells, the similar decrease in cell viability caused by 75 μM SIN-1 was not protected to any significant extent by D3T pretreatment (Figure 7(C),(D)). As shown above in Figure 3(B), cotreatment of RINm5F cells with 25 μM BSO led to a complete abolishment of the D3T-induced elevation of cellular GSH.

Discussion

The glutathione system has been suggested to play important roles in protecting against oxidative injury in pancreatic beta-cells [13–15]. Thus, investigation

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

RIGHTSLINK

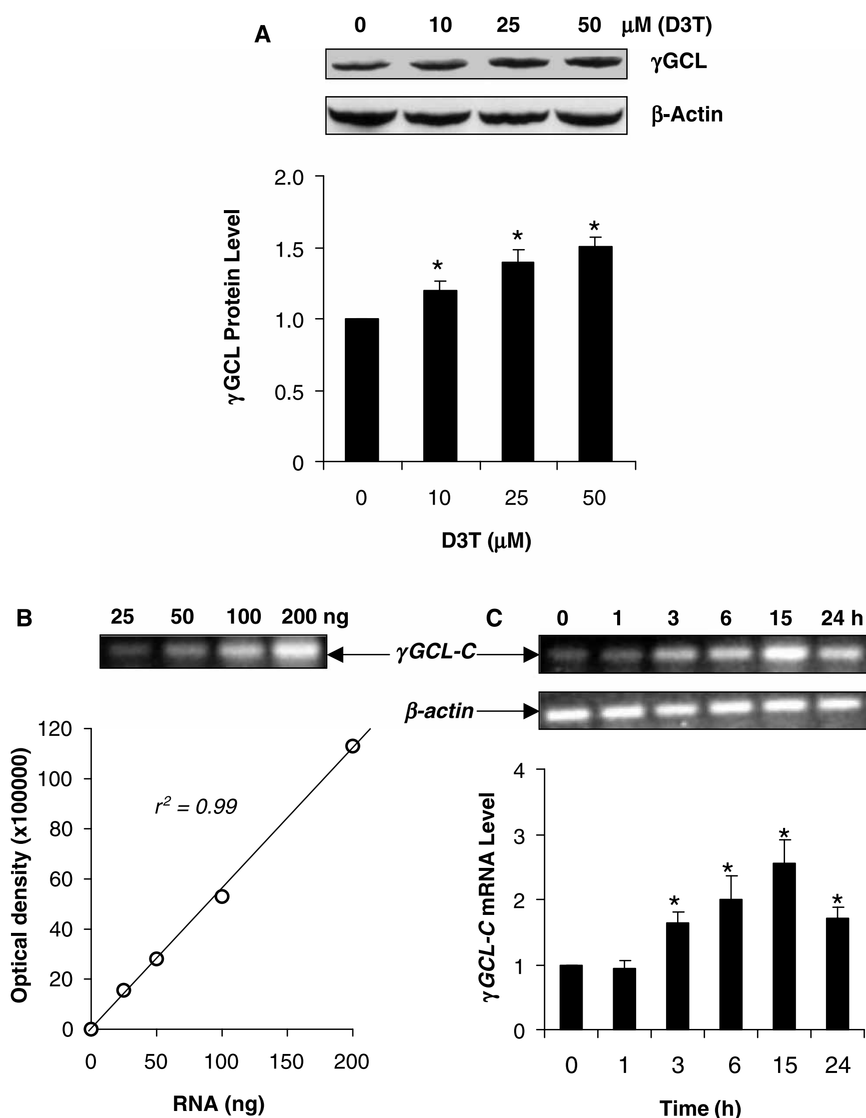

Figure 4. Effects D3T on protein and mRNA expression of γGCL in RINm5F cells. In (A), cells were incubated with the indicated concentrations of D3T for 24 h, followed by determination of the protein expression of γGCL and β-actin by immunoblot. The anti-γGCL antibody recognized the catalytic subunit of the γGCL protein. In (B), gel picture and line graph showing linear amplification of the PCR product of γGCL-C mRNA. In (C), representative gel pictures showing γGCL-C and β-actin mRNA expression at different time points after treatment with 50 μM D3T; bar graph showing quantitative analysis of the γGCL-C mRNA expression. Values in (A) and (C) represent means ± SEM from three independent experiments. *, significantly different from 0 μM D3T (A) or 0 h (C). β-Actin serves as a sample loading control.

of the chemical regulation of endogenous glutathione system in beta-cells is of importance for developing novel chemoprotective strategies for intervention of the oxidative degeneration of pancreatic beta-cells in diabetes. The results of this study demonstrated that the endogenous glutathione system (GSH, GR and GPx) in pancreatic RINm5F cells could be significantly upregulated by D3T, a potent chemoprotectant extensively utilized for cancer chemoprotection [27]. The inducibility of these cytoprotective factors in beta-cells had not been previously investigated. Although RINm5F cells expressed extreme low activity of GPx similar to that observed with normal rat pancreatic islets, this enzyme was highly inducible by D3T (Figure 1), indicating that GPx in beta-cells is subject to chemical regulation. The high basal expression of GR in RINm5F cells observed in this study (Figure 1) was also in line with a previous report that normal rat pancreatic islets constitutively expressed a high level of GR [28]. It remains unclear why GR is highly expressed in pancreatic islet cells while other antioxidants, including SOD, catalase, and GPx are relatively deficient in these cells [6,7]. It has been suggested that the *de novo* synthesis of GSH in beta-cells proceeds at low levels, and the high GR activity in these cells may thus help maintain the GSH level via reducing the oxidized form of glutathione (GSSG) to GSH [28]. In contrast to the high

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

RIGHTS LINK

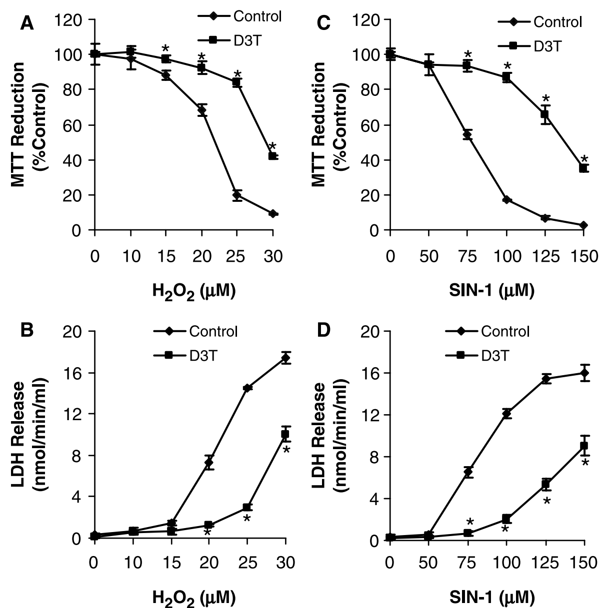

Figure 5. Protective effects of D3T pretreatment on H_2O_2 (A, B) or SIN-1 (C, D)-mediated cytotoxicity in RINm5F cells. Cells were pretreated with 50 μM D3T for 24 h, followed by incubation with the indicated concentrations of H_2O_2 or SIN-1 for another 24 h. After this incubation, cytotoxicity was determined using MTT reduction and LDH release assay. Values represent means ± SEM from 3–4 independent experiments. *, significantly different from the respective control.

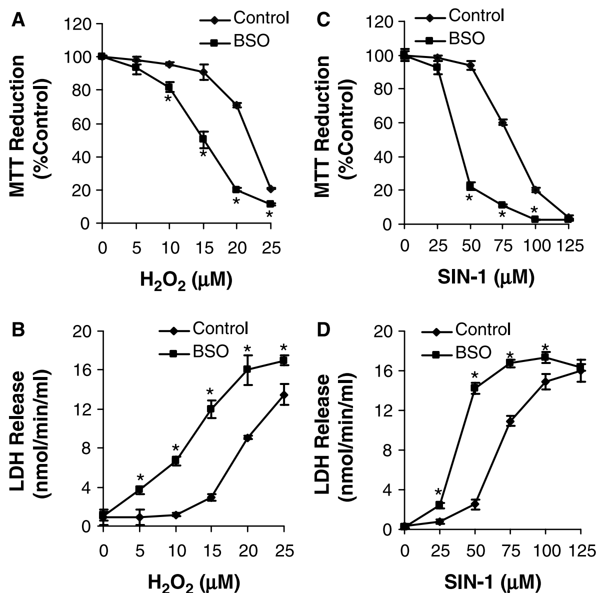

Figure 6. Potentiation of H_2O_2 (A, B) or SIN-1 (C, D)-induced cytotoxicity by BSO pretreatment in RINm5F cells. Cells were pretreated with 25 μM BSO, and then incubated with the indicated concentrations of H_2O_2 or SIN-1 for another 24 h. After this incubation cytotoxicity was determined using MTT reduction and LDH release assay. Values represent means ± SEM from 3–4 independent experiments. *, significantly different from respective control.

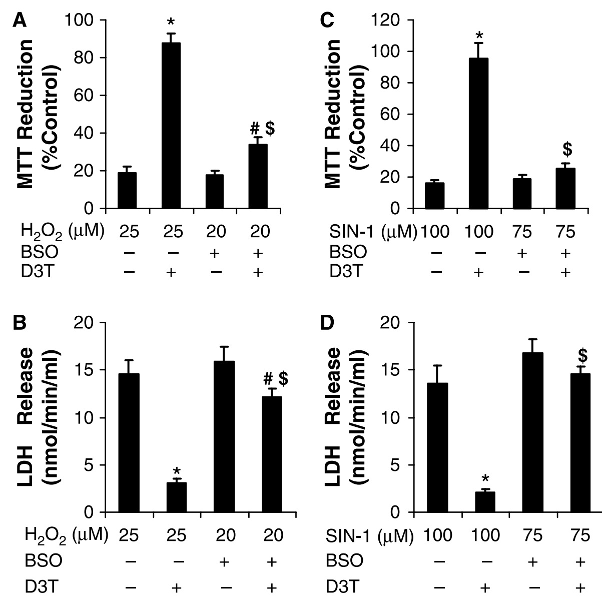

Figure 7. The effects of cotreatment with BSO on D3T-mediated protection against H_2O_2 (A, B) or SIN-1 (C, D)-induced cytotoxicity in RINm5F cells. Cells were pretreated with 25 μM BSO, or 25 μM BSO + 50 μM D3T for 24 h. The media were then removed, and cells were further incubated with the indicated concentrations of H_2O_2 or SIN-1 for another 24 h, followed by determination of cytotoxicity using MTT reduction and LDH release assay. Values represent means ± SEM from three separate experiments. In (A) and (B), *, significantly different from 25 μM H_2O_2 group; #, significantly different from 20 μM H_2O_2 + BSO group; $, significantly different from 25 μM H_2O_2 plus D3T group. In (B), *, significantly different from 100 μM SIN-1 group; $, significantly different from 100 μM SIN-1 plus D3T group.

inducibility of GSH and GPx by D3T, GR was only slightly induced by D3T in RINm5F cells (Figure 1). This might be due to the expression of GR gene in this beta-cell line approaching a maximal level under unstimulated conditions. Nevertheless, the similarity between RINm5F cells and normal rat pancreatic islet cells in terms of expression of glutathione system, as well as the inducibility of GSH, GR, and GPx in RINm5F cells make this cell line a useful model for studying beta-cell expression of glutathione system and its regulation by chemoprotectants.

One notable observation of this study was the remarkable induction of GSH by D3T in RINm5F cells (Figures 1 and 2). GSH is synthesized in cytoplasm via 2 consecutive enzymatic reactions [26]. The key enzyme is γGCL, which can be selectively and patently inhibited by BSO [26]. As such, BSO is widely used as a chemical tool to manipulate cellular GSH level. The complete reversion of the D3T-mediated elevation of GSH by BSO cotreatment suggested that D3T-mediated GSH induction occurred via a γGCL-dependent process. This notion was further supported by the observation that D3T also increased the protein expression of γGCL and the mRNA level of γGCL-C (Figure 4). Activation of gene expression for γGCL as well as several

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

RIGHTSLINK

GSH-linked enzymes by D3T has also been previously demonstrated with other types of cells, including bone marrow stromal and cardiac cells [18,22,23,29]. Furthermore, Nrf2 signaling has been shown to be crucially involved in D3T-mediated induction of GSH system in the above cell types [22,29]. It is likely that Nrf2 signaling may also be involved in the upregulation of GSH system by D3T in RINm5F beta-cells.

Because of the ability of D3T treatment to upregulate cellular GSH, GR, and GPx, which are important cellular defenses against oxidative stress, we investigated whether induction of the above cytoprotective factors by D3T led to protection against ROS/RNS-mediated cytotoxicity in RINm5F cells. The results of the present study clearly showed that pretreatment of RINm5F cells with D3T afforded a remarkable protection against H_2O_2 or SIN-1-elicted cytotoxicity (Figure 5). SIN-1 autooxidizes under a physiological pH to produce both superoxide and nitric oxide, which react at a diffusion-limited rate to form peroxynitrite, a highly toxic RNS. As such, SIN-1 is commonly used for studying the biological effects of peroxynitrite [18,22,29]. While a number of antioxidants are involved in H_2O_2 detoxification, GSH/GPx has been demonstrated to be largely responsible for conversation of H_2O_2 to water in mammalian cells [24]. In addition to being involved in detoxifying H_2O_2, GPx/GSH is also found to be a major pathway for detoxification of peroxynitrite in mammalian cells [18,25]. Thus, the potent induction of GSH and GPx by D3T may largely contribute to the increased resistance of the D3T-pretreated RINm5F cells to the above ROS/RNS-mediated cytotoxicity (Figure 5). Furthermore, the concurrent induction of GR by D3T may lead to increased regeneration of GSH from GSSG produced during the GPx-catalyzed decomposition of H_2O_2 or peroxynitrite in RINm5F cells.

To further investigate the involvement of GSH in protecting against ROS/RNS-elicited cell injury, the cytotoxicity was determined in GSH-depleted cells. The dramatic potentiation of H_2O_2 or SIN-1-induced cytotoxicity by BSO pretreatment (Figure 6) indicated that GSH was a crucial cellular defense against the ROS/RNS-mediated toxicity in RINm5F cells. To provide direct evidence for the causal involvement of GSH in D3T-mediated cytoprotection against the ROS/RNS-induced toxicity, RINm5F cells were pretreated with D3T + BSO followed by exposure to the ROS/RNS. Cotreatment with BSO prevented D3T-induced GSH elevation (Figure 3(B)), and remarkably ablated the D3T-mediated cytoprotetion (Figure 7). The complete reversion by BSO-cotreatment of D3T cytoprotection against SIN-1-mediated toxicity (Figure 7(C),(D)) indicated that upregulation of GSH was the predominant mechanism underlying D3T-mediated cytoprotection against peroxynitrite toxicity in the beta-cells. On the other hand, the non-complete reversion of H_2O_2-induced cell injury by BSO-cotreatment (Figure 7(A),(B)) suggested that other D3T-inducible cytoprotective factors might also contribute to D3T cytoprotection against H_2O_2-elicited toxicity in RINm5F cells.

In summary, this study conclusively demonstrated that D3T treatment of RINm5F cells resulted in a significant induction of cellular GSH, GR, and GPx, and augmentation of mRNA and protein expression of γGCL. This study also revealed that upregulation of the endogenous GSH biosynthesis by D3T appeared to be a principal mechanism underlying D3T-mediated cytoprotection against ROS/RNS-elicited toxicity in the pancreatic beta-cell line. This study thus demonstrated a novel strategy for protecting against oxidative *in vitro* beta-cell injury via chemoprotectant-mediated upregulation of endogenous glutathione system.

Acknowledgements

This work was supported in part by NIH grant HL71190 (Y.L.). M.A.T. was supported by NIH grants ES03760, ES03819 and ES08078.

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

References

[1] Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: Principles of pathogenesis and therapy. Lancet 2005;365: 1333–1346.

[2] Mathis D, Vence L, Benoist C. Beta-cell death during progression to diabetes. Nature 2001;414:792–798.

[3] Rhodes CJ. Type 2 diabetes: A matter of beta-cell life and death? Science 2005;307:380–384.

[4] Robertson RP. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J Biol Chem 2004;279:42351–42354.

[5] Kaneto H, Kawamori D, Matsuoka TA, Kajimoto Y, Yamasaki Y. Oxidative stress and pancreatic beta-cell dysfunction. Am J Ther 2005;12:529–533.

[6] Grankvist K, Marklund SL, Taljedal IB. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem J 1981;199:393–398.

[7] Lenzen S, Drinkgern J, Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. Free Radic Biol Med 1996;20:463–466.

[8] Donath MY, Storling J, Maedler K, Mandrup-Poulsen T. Inflammatory mediators and islet beta-cell failure: A link between type 1 and type 2 diabetes. J Mol Med 2003;81: 455–470.

[9] Tabatabaie T, Vasquez-Weldon A, Moore DR, Kotake Y. Free radicals and the pathogenesis of type 1 diabetes: Beta-cell cytokine-mediated free radical generation via cyclooxygenase-2. Diabetes 2003;52:1994–1999.

[10] Rahimi R, Nikfar S, Larijani B, Abdollahi M. A review on the role of antioxidants in the management of diabetes and its complications. Biomed Pharmacother 2005;59:365–373.

[11] 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,

RIGHTSLINK

prevents autoimmune and streptozotocin-induced diabetes. J Exp Med 1998;188:1445–1451.

[12] Lortz S, Tiedge M, Nachtwey T, Karlsen AE, Nerup J, Lenzen S. Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. Diabetes 2000;49:1123–1130.

[13] Tanaka Y, Tran PO, Harmon J, Robertson RP. A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. Proc Natl Acad Sci USA 2002;99:12363–12368.

[14] Tran PO, Parker SM, LeRoy E, Franklin CC, Kavanagh TJ, Zhang T, Zhou H, Vliet P, Oseid E, Harmon JS, Robertson RP. Adenoviral overexpression of the glutamylcysteine ligase catalytic subunit protects pancreatic islets against oxidative stress. J Biol Chem 2004;279:53988–53993.

[15] Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H. Glucose toxicity in beta-cells: Type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes 2003;52:581–587.

[16] Zhu H, Li Y, Trush MA. Differences in xenobiotic detoxifying activities between bone marrow stromal cells from mice and rats: Implications for benzene-induced hematotoxicity. J Toxicol Environ Health 1995;46:183–201.

[17] Otieno MA, Kensler TW, Guyton KZ. Chemoprotective 3*H*-1,2-dithiole-3-thione induces antioxidant genes *in vivo*. Free Radic Biol Med 2000;28:944–952.

[18] Cao Z, Li Y. Protecting against peroxynitrite-mediated cytotoxicity in vascular smooth muscle cells via upregulating endogenous glutathione biosynthesis by 3*H*-1,2-dithiole-3-thione. Cardiovasc Toxicol 2004;4:339–353.

[19] Gurgul E, Lortz S, Tiedge M, Jorns A, Lenzen S. Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. Diabetes 2004;53:2271–2280.

[20] Yang F, Bleich D. Transcriptional regulation of cyclooxygenase-2 gene in pancreatic beta-cells. J Biol Chem 2004;279: 35403–35411.

[21] Wang H, Horikawa Y, Jin L, Narita T, Yamada S, Shihara N, Tatemoto K, Muramatsu M, Mune T, Takeda J. Gene expression profile in rat pancreatic islet and RINm5F cells. J Mol Endocrinol 2005;35:1–12.

[22] Zhu H, Itoh K, Yamamoto M, Zweier JL, Li Y. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: Protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett 2005;579: 3029–3036.

[23] Li Y, Cao Z, Zhu H, Trush MA. Differential roles of 3*H*-1,2-dithiole-3-thione-induced glutathione, glutathione S-transferase and aldose reductase in protecting against 4-hydroxy-2-nonenal toxicity in cultured cardiomyocytes. Arch Biochem Biophys 2005;439:80–90.

[24] Kehrer JP. Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol 1993;23:21–48.

[25] Sies H, Sharov VS, Klotz LO, Briviba K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations: A new function for selenoproteins as peroxynitrite reductase. J Biol Chem 1997;272:27812–27817.

[26] Anderson ME. Glutathione: An overview of biosynthesis and modulation. Chem Biol Interact 1998;111–112:1–14.

[27] Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K, Yamamoto M, Kensler TW. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. Mutat Res 2001;480–481:305–315.

[28] Nagaoka Y, Iuchi Y, Ikeda Y, Fujii J. Glutathione reductase is expressed at high levels in pancreatic islet cells. Redox Rep 2004;9:321–324.

[29] Zhu H, Zhang L, Itoh K, Yamamoto M, Ross D, Trush MA, Zweier JL, Li Y. Nrf2 controls bone marrow stromal cell susceptibility to oxidative and electrophilic stress. Free Radic Biol Med 2006;41:132–143.

Free Radic Res Downloaded from informahealthcare.com by University of Saskatchewan on 12/02/11
For personal use only.

RIGHTSLINK
Copyright Clearance Center