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Inhibition of NO-induced β-cell death by novel NF-κB inhibitor (−)-DHMEQ via activation of Nrf2–ARE pathway

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ABSTRACT

Excessive nitric oxide (NO) plays a pivotal role in the progression of β-cell apoptosis in type 1 diabetes mellitus. We used mouse insulinoma Min6 cells as a model of β cells in this research. We found that (−)-DHMEQ, an NF-κB inhibitor, rescued β cells from NO-induced apoptosis, and then studied the mechanism of apoptosis inhibition. (−)-DHMEQ activated Nrf2 and induced transcription of Nrf2-target genes following the increase of antioxidant response element (ARE) reporter activity. Similarly, *tert*-butyl hydroquinone (tBHQ), a known activator of Nrf2, inhibited NO-induced cell death along with the transcriptional activation of ARE. RNAi-mediated knockdown of Nrf2 lowered the cytoprotective effect of (−)-DHMEQ against NO, suggesting that (−)-DHMEQ inhibited NO-induced cell death via Nrf2 activation. Furthermore, overexpression of Nrf2 rendered cells to be more resistant to NO, indicating that Nrf2 activation provides critical defense function against NO in Min6 cells. Taken together, we conclude that (−)-DHMEQ may be a useful therapeutic agent for type 1 diabetes mellitus in the onset of disease by protecting β cells from apoptosis.

1. Introduction

Type 1 diabetes mellitus is characterized by severe insulin deficiency resulting from chronic and progressive destruction of pancreatic β cells by the immune system [1]. Beta-cell destruction has been reported to occur in various stimuli including pro-inflammatory cytokines, such as IL-1β, IFN-γ and TNF-α, and reactive oxygen species, such as nitric oxide (NO). These stimuli are known to induce β-cell death, which is mainly in the form of apoptosis [2]. Several studies have indicated that excessive NO released by islet-infiltrating immune cells or cytokine-exposed β cells themselves plays a pivotal role in the progression of β-cell apoptosis. Indeed, rat treated with inducible nitric oxide synthase (iNOS) inhibitor is known to reduce the incident of diabetes mellitus [3]. Moreover, microarray analysis revealed that nearly 50% of the cytokine-modified genes are secondary to NO formation [4].

Nuclear factor κB (NF-κB) is a transcription factor which regulates the expression of numerous genes important for cell growth, survival, and inflammatory response [5]. NF-κB exists as homo- or heterodimers of five related Rel-family proteins: RelA/p65, RelB, c-Rel, p50 (a processing product of p105) and p52 (a processing product of p100). The predominant species involved in β-cell death is related to be p65-p50 heterodimer [6–9]. Under normal condition, NF-κB (p65-p50 heterodimer) is bound to inhibitor κB (IκB) and remained in the cytoplasm. After stimulation by various agents to induce degradation of IκB, NF-κB translocates to the nucleus and induces downstream gene expression. Although NF-κB is known to regulate antiapoptotic gene expression in diverse cell types, NF-κB activation is mostly proapoptotic in β cells. Indeed, cytokine-induced β-cell death is inhibited by transfection with a dominant negative or nondegradable mutant form of IκB [6–8]. Furthermore, NF-κB also plays an important proapoptotic role in β cells in vivo [9]. Thus, NF-κB is a key regulator of β-cell death in type1 diabetes.

NF-E2-related Factor 2 (Nrf2) is a transcription factor which plays a central role in inducible expression of numerous detoxifying and antioxidant genes, such as heme oxygenase-1 (HO-1), NAD (P) H quinine oxidoreductase 1 (NQO1) and glutamate cysteine ligase catalytic subunit (GCLC) [10]. Under normal condition, Nrf2 is constantly ubiquitinated by the Cul3-Keap1 (Kelch ECH associating protein 1) ubiquitin E3 ligase complex and subsequently degraded. Upon exposure to electrophilic and oxidative stresses, Nrf2 is liberated from Keap1-mediated repression due to impairment of structural integrity of Keap1–Nrf2 complex [11]. Then, Nrf2 quickly

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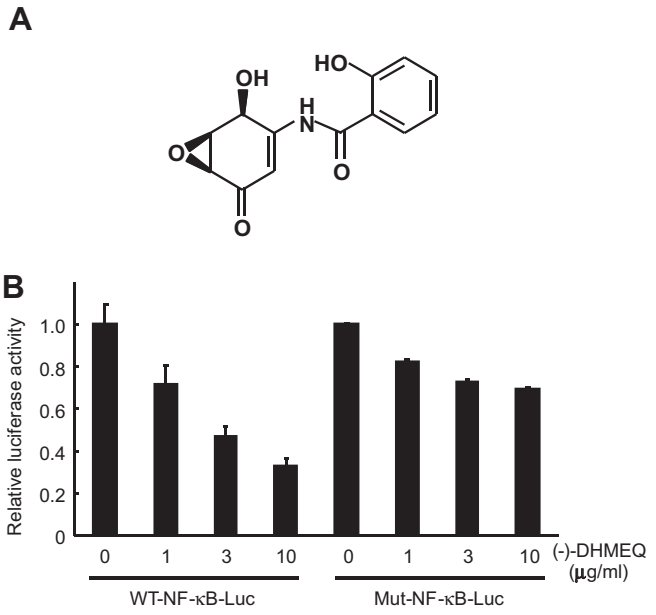

Fig. 1. Inhibition of NF-κB activity by (−)-DHMEQ in Min6 cells. (A) Chemical structure of (−)-DHMEQ. (B) Inhibition of NF-κB activity by (−)-DHMEQ. Cells were transfected with wild-type or mutant NF-κB luciferase reporter plasmid and phRL TK plasmid. After 24 h, cells were treated with the indicated concentrations of (−)-DHMEQ for another 24 h. Then, the luciferase activity was measured.

translocates to the nucleus and binds to the antioxidant responsive element (ARE), resulting in the transcriptional induction of several dozen cytoprotective genes. The analyses of *nrf2*-null mutant mice have revealed that the genes regulated by Nrf2 are indispensable components of defense mechanism against oxidative stresses [10]. Since β cells are known to be susceptible to oxidative stresses due to their low expression of antioxidant enzymes [12], activation of Nrf2 could be a potential effective strategy for β-cell protection. Recently, several chemopreventive agents were reported to inhibit NF-κB signaling with a concomitant activation of Nrf2 signaling pathway [13,14]. However, cytoprotective role of Nrf2 and these chemopreventive agents in β cells still remains unclear.

Previously, we have designed and synthesized a potent NF-κB inhibitor, (−)-dehydroxymethylepoxyquinomycin ((−)-DHMEQ, Fig. 1A) [15]. This compound inhibits NF-κB activity via covalently binding to highly conserved cysteine residue in Rel family proteins [16]. In the present study, we found that (−)-DHMEQ inhibited NO-induced β-cell death. Mechanistic study revealed that (−)-DHMEQ exhibited the cytoprotective effect via activation of Nrf2–ARE pathway.

2. Materials and methods

2.1. Materials

(−)-DHMEQ was synthesized in our laboratory as described previously [15].

S-nitroso-*N*-acetyl-*DL*-penicillamine (SNAP) and *tert*-butyl hydroquinone (tBHQ) were purchased from Cayman Chemical Company (Ann Arbor, MN) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. Anti-Nrf2 antibody was purchased from Santa Cruz Biotechnology (St. Louis, MO).

2.2. Cell culture

Min6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 25 mM glucose, 15% (v/v) heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 units/ml penicillin G (Sigma, St. Louis, MO), 200 μg/ml kanamycin (Sigma, St. Louis, MO), 600 μg/ml L-glutamine (Sigma, St. Louis, MO), and 79 μM 2-mercaptoethanol in humidified 5% CO_2/95% air at 37 °C.

2.3. Trypan blue dye exclusion assay

After chemical treatment, cells were collected by trypsinization, and then combined with cells floating in the medium. Cells were subsequently stained with trypan blue dye, and the number of total and stained cells was counted.

2.4. Griess assay

Fresh cultured medium (200 μl) was added in each well of 96-well plate (Corning, Corning, NY). After chemical treatment, 100 μl of Griess reagent was added to the equal volumes of suspension from each well. The concentration of NO was obtained as accumulated nitrite in the medium by measuring the absorbance at 570 nm with a microplate reader.

2.5. Apoptosis assay

Apoptosis was analyzed using the APOPercentage™ assay kit according to the manufacturer's instructions with minor modification. Cells were seeded in a 48-well plate (Corning, Corning, NY) without using Gelatin Matrix forming solution. One hour before the chemical incubation time was reached, medium was removed and replaced 250 μl of fresh culture medium supplemented with 12.5 μl of APOPercentage™ dye. Then, the cells were photographed after washing twice with PBS. When quantizing the ratio of apoptotic cells, cells were collected by trypsinization, and then combined with cells floating in the medium supplemented with 5% (v/v) APOPercentage™ dye. Thereafter, the number of total and stained cells was counted.

2.6. Luciferase reporter assay

Both wild-type and mutant NF-κB luciferase reporter plasmids were constructed in reference to the sequence information described in literature [17]. The wild-type ARE luciferase reporter plasmid was constructed as described previously [18]. Transfection of plasmid DNA was performed by using Lipofectamine™ LTX (Invitrogen, Carlsvad, CA) and PLUS™ reagent (Invitrogen, Carlsvad, CA). After 24 h, the cells were treated with chemicals for another 24 h, and then the luciferase activity was measured. The levels of firefly luciferase activity were normalized to that of *Renilla* luciferase activity for each transfection.

2.7. RT–PCR

After the chemical treatment, total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA). Then, 1 μg of mRNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kits (Life Technology, Carlsbad, CA), and 2 μl of the cDNA mixture was used for enzymatic amplification using TaKaRa Taq™ (Takara Bio, Shiga, Japan). Primer sequences used for semi-quantitative RT–PCR were as follows; HO-1, 5′-CACGCATATACCCGCTACCT-3′ (forward) and 5′-CCAGAGTGTTCATTCGAGCA-3′ (reverse); GCLC, 5′-CACTGCCAGAACACAGACCC-3′ (forward) and 5′-ATGGTCTGGCTGAGAAGCCT-3′ (reverse); NQO1, 5′-GCAGGATTTGCCTACACAATATGC-3′ (forward) and 5′-AGTGGTGATAGAAAGCAAGGTCTTC-3′ (reverse); Nrf2, 5′-TGGACGGGACTATTGAAGGCTG-3′ (forward) and 5′-CGCCTTTTCAGTAGATGGAGG-3′ (reverse); β-actin, 5′-CTTCGAGCAGGAGATGGCCA-3′ (forward) and 5′-CCAGACAGCACTGTGTTGGC-3′ (reverse).

2.8. Western blotting

After chemical treatment, cells were lysed with lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and the protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)). Cell lysates were subjected to immunoblotting with the indicated antibodies and the immune complexes were detected with Immobilon Western chemiluminescence HRP substrate (Millipore, Billerica, MA).

2.9. Knockdown of Nrf2 by siRNA

Transfection of siRNA was performed by using Lipofectamine™ RNAiMAX Reagent (Invitrogen, Carlsvad, CA). After 24 h, medium was removed and replaced fresh antibiotic free medium, and then incubated for another 24 h. When performing typan blue dye exclusion assay, cells were then treated with chemicals. Primer sequences used for control-siRNA were as follows; 5′-CGUACGCG-GAAUACUUCGAdTdT-3′ (forward) and 5′-UCGAAGUAUUCCGC-GUACGdTdT-3′(reverse). Nrf2 siRNA (sc-37049) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.10. Plasmid construction and transfection

Mouse Nrf2 cDNA was inserted into pCMV-Tag2B vector by using two step procedures. First, Nrf2 fragment A (1–969) containing HindIII site near the 3′-terminus (950–955) was cloned to PstI-XhoI sites of pCMV-Tag2B vector. Then, Nrf2 fragment B (920–1794) containing HindIII site near the 5′-terminus (950–955) was cloned to HindIII-XhoI sites of pCMV-Tag2B-Nrf2 (1–969) vector. Both Nrf2 fragments were generated by PCR amplification of mouse cDNA. Primer sequences used for preparation of

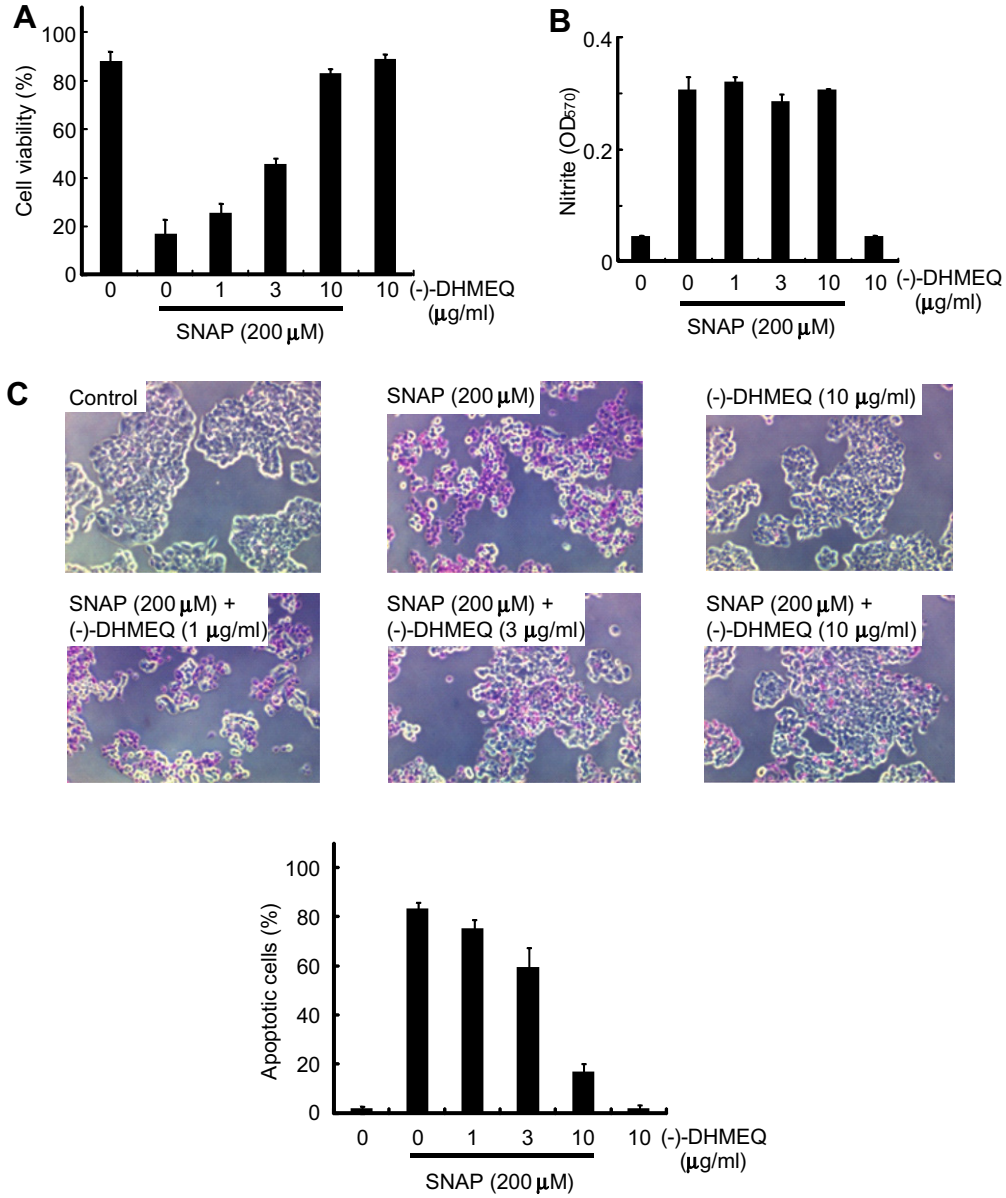

Fig. 2. Inhibition of NO-induced β-cell apoptosis by (−)-DHMEQ. (A) Inhibition of SNAP-induced β-cell death by (−)-DHMEQ. Cells were pretreated with the indicated concentrations of (−)-DHMEQ for 2 h, and then treated with 200 μM SNAP for 12 h. Cell viability was assessed by the trypan blue dye exclusion assay. (B) Effect of (−)-DHMEQ on NO release by SNAP. (−)-DHMEQ was added in medium at the indicated concentrations for 2 h, and then treated with 200 μM SNAP for 12 h. NO production was assessed by the Griess assay. (C). Inhibition of SNAP-induced β-cell apoptosis by (−)-DHMEQ. Cells were pretreated with the indicated concentrations of (−)-DHMEQ for 2 h, and then treated with 200 μM SNAP for 12 h. Thereafter, the cells were stained by APOPercentage™ dye and photographed (upper) and quantified (lower).

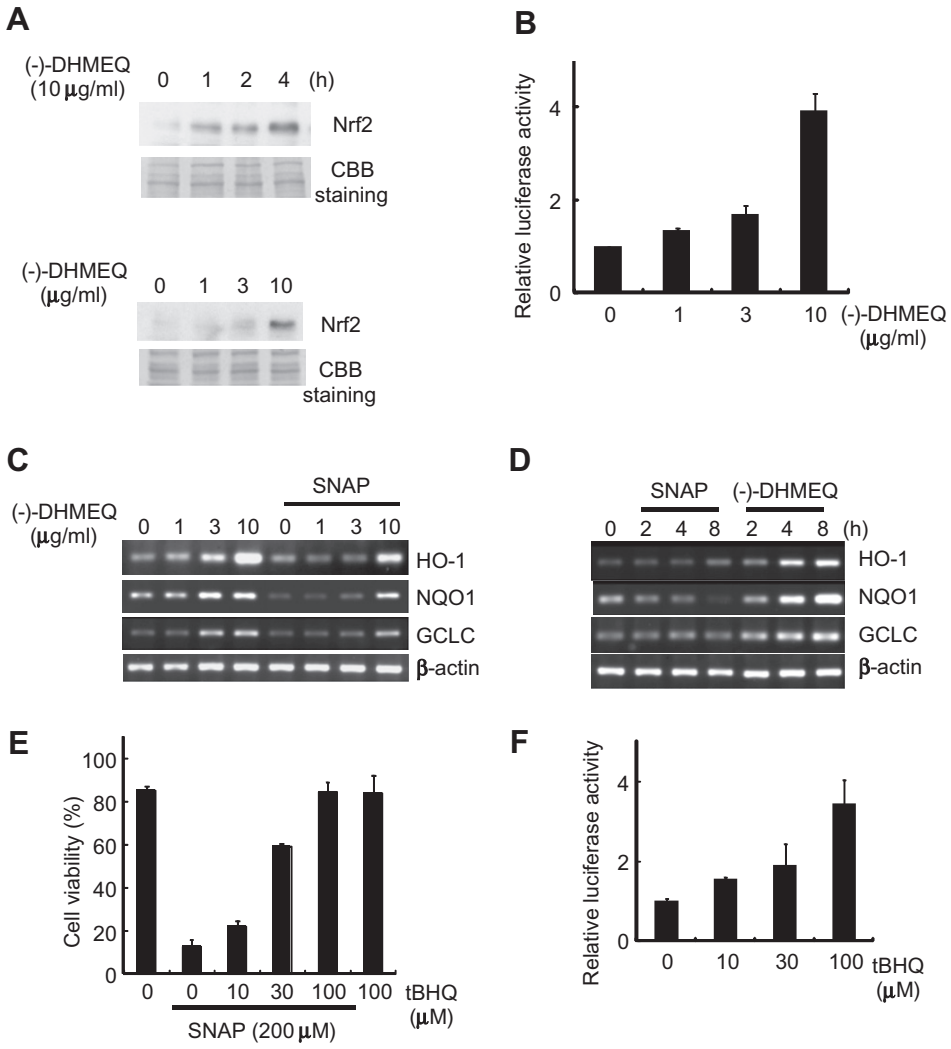

Fig. 3. Activation of Nrf2–ARE signaling pathway by (−)-DHMEQ in β cells. (A) Increase in Nrf2 protein levels by (−)-DHMEQ. (Upper) Cells were treated with 10 μg/ml (−)-DHMEQ for the indicated periods. Then, total cell lysates were assessed by Western blotting. (Lower) Cells were treated with the indicated concentrations of (−)-DHMEQ for 4 h. Then, total cell lysates were assessed by Western blotting. (B) Induction of ARE luciferase reporter activity by (−)-DHMEQ. Cells were transfected with wild-type ARE luciferase reporter plasmid and phRL TK plasmid. After 24 h, cells were treated with the indicated concentrations of (−)-DHMEQ for another 24 h. Then, the luciferase activity was measured. (C) Dose-dependent induction of Nrf2-target genes by (−)-DHMEQ. Cells were pretreated with the indicated concentrations of (−)-DHMEQ for 2 h, and then treated with or without 200 μM SNAP for 2 h. Thereafter, total cell lysates were assessed by RT–PCR. (D) Time-course analysis of Nrf2-target genes during treatment with SNAP or (−)-DHMEQ. Cells were treated with 200 μM SNAP or 10 μg/ml (−)-DHMEQ for the indicated periods. Then, total cell lysates were assessed by RT–PCR. (E) Inhibition of SNAP-induced β-cell death by tBHQ. Cells were pretreated with the indicated concentrations of tBHQ for 2 h, and then treated with 200 μM SNAP for 12 h. Cell viability was assessed by the trypan blue dye exclusion assay. (F) Induction of ARE luciferase reporter activity by tBHQ. Cells were transfected with wild-type ARE luciferase reporter plasmid and phRL TK plasmid. After 24 h, cells were treated with the indicated concentrations of tBHQ for another 24 h. Then, the cell lysate was collected and the luciferase activity was measured.

Nrf2 cDNA fragments were as follows; Nrf2 fragment A (1–969), 5′-TTTTCTGCAGATGATGGACTTGGAGTTGCC-3′ (forward) and 5′-TTTTCTCGAGGTGCTTCGGGTTGAAAGCTT-3′ (reverse); Nrf2 fragment B (920–1794), 5′-CTATTGAAGGCTGTGACCTGTCACTGTGTA-3′ (forward) and 5′-TTTTCTCGAGCTAGTTTTTCTTTGTATCTG-3′ (reverse). Transfection of plasmid DNA was performed by using Lipofectamine™ LTX (Invitrogen, Carlsvad, CA) and PLUS™ reagent (Invitrogen, Carlsvad, CA). After 16 h, medium was removed and replaced fresh antibiotic free medium, and then incubated for another 32 h. When performing typan blue dye exclusion assay, cells were then treated with chemicals.

3. Results

3.1. Inhibition of NO-induced β-cell apoptosis by (−)-DHMEQ

(−)-DHMEQ strongly inhibited endogenous activity of NF-κB in Min6 cells, just as did in other cell types (Fig. 1B). Pretreatment of (−)-DHMEQ significantly protected Min6 cells from an NO-donor SNAP-induced toxicity (Fig. 2A). To exclude the possibility that this effect is due to the direct NO-scavenge by (−)-DHMEQ, we analyzed the amount of nitrite in the presence of (−)-DHMEQ. Griess assay demonstrated that (−)-DHMEQ did not inhibit formation of nitrite by SNAP (Fig. 2B). We next examined whether (−)-DHMEQ inhibits NO-induced apoptosis by using the dye selectively imported in the apoptotic cells. As shown in Fig. 2C, treatment with (−)-DHMEQ significantly reduced levels of apoptosis. Taken together, these findings indicate that (−)-DHMEQ inhibited NO-induced apoptosis in Min6 cells.

3.2. Activation of Nrf2–ARE signaling pathway by (−)-DHMEQ

Nrf2 is a key transcription factor regulating the induction of cytoprotective enzymes. We then studied whether (−)-DHMEQ activates Nrf2–ARE signaling pathway. Treatment with (−)-DHMEQ

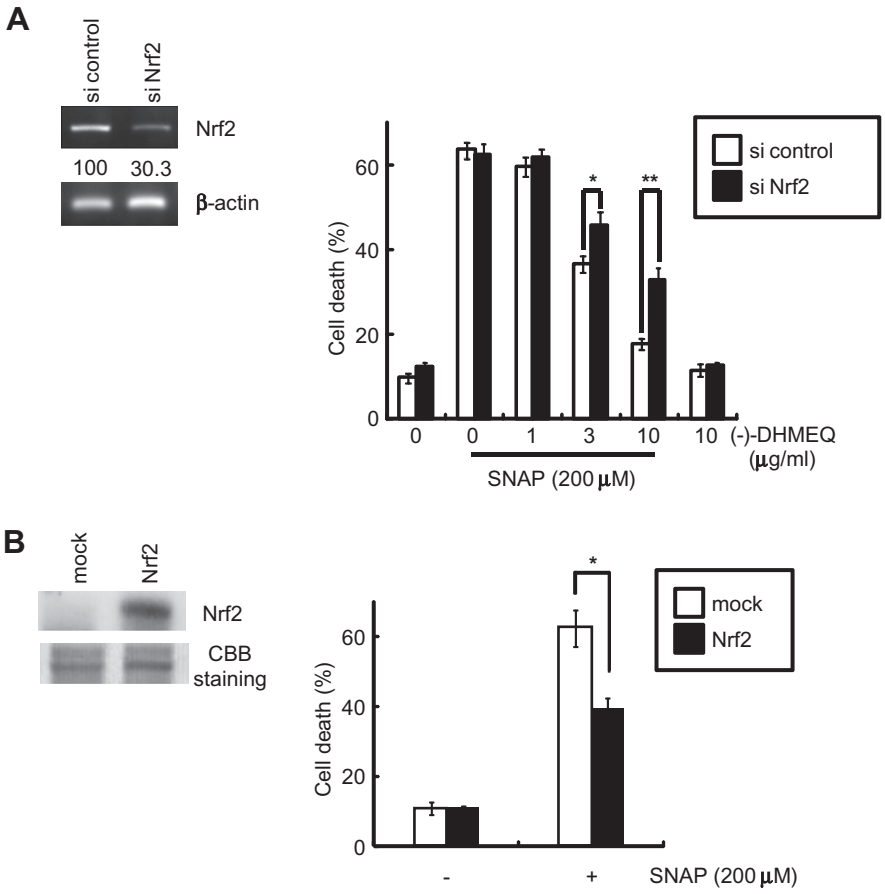

Fig. 4. Requirement of Nrf2 for cytoprotective responses against NO in β cells. (A) Inhibition of cytoprotective effect of (−)-DHMEQ against NO by knockdown of Nrf2 in β cells. Cells were transfected with indicated siRNA at a final concentration of 100 nM. After 48 h, total cell lysates were assessed by RT–PCR (left) or cell viability was assessed by the trypan blue dye exclusion assay following chemical treatment (right). $^{*}p < 0.05$ as indicated; $^{**}p < 0.005$ as indicated. (B) Inhibition of SNAP-induced β-cell death by Nrf2 overexpression. Cells were transiently transfected with empty vector or Nrf2 expression vector for 48 h. After the transfection period, total cell lysates were assessed by Western blotting (left) or cell viability was assessed by the trypan blue dye exclusion assay following chemical treatment (right). $^{*}p < 0.01$ as indicated.

resulted in accumulation of Nrf2 protein (Fig. 3A). Next, the cells were transfected with ARE luciferase reporter construct. As shown in Fig. 3B, (−)-DHMEQ increased ARE-driven luciferase activity. Moreover, (−)-DHMEQ also induced mRNA expressions of Nrf2-downstream genes including HO-1, NQO1 and GCLC even in the presence of NO (Fig. 3C). Augmentation of these enzyme expressions by (−)-DHMEQ remained at least up to 8 h (Fig. 3D). To further validate the effect of Nrf2 activation on NO-induced cell death, cells were treated with *tert*-butyl hydroquinone (tBHQ), a known Nrf2 activator. As shown in Fig. 3E, tBHQ significantly inhibited NO-induced cell death. In parallel with this observation, treatment with tBHQ increased the ARE-driven luciferase activity (Fig. 3F). These results indicate that Nrf2 activators, including (−)-DHMEQ, have the potential to protect Min6 cells from NO.

3.3. Requirement of Nrf2 for cytoprotective responses against NO in β cells

To further confirm that the protective effect of (−)-DHMEQ is indeed mediated through Nrf2 activation, cells were treated with Nrf2-siRNA construct. As shown in Fig. 4A, cytoprotective effect of (−)-DHMEQ was significantly lowered in Nrf2-siRNA transfected cells compared to control-siRNA transfected cells. In a complementary manner to the results of siRNA experiment, the expression vector of Nrf2 was transiently transfected into Min6 cells. As shown in Fig. 4B, Nrf2-overexpression rendered cells to be more resistant against NO. These data indicate that Nrf2 is a key component of antiapoptotic signaling in NO-exposed Min6 cells.

4. Discussion

Type 1 diabetes mellitus is caused by autoimmune destruction of β cells mainly in the form of apoptosis [2]. Therefore, discovery of pharmaceutical agents aimed at inhibiting β-cell apoptosis should be useful for treatment of type 1 diabetes mellitus. In the present study, we found that an NF-κB inhibitor (−)-DHMEQ inhibited NO-induced apoptosis in mouse insulinoma Min6 cells (Fig. 2C). Although NF-κB plays an important proapoptotic role in β-cell dysfunction induced by various cytotoxic agents [6–9], Tonnesen et al. reported that NO does not cause NF-κB activation in β cells [19]. Thus, the role of NF-κB in NO-induced β-cell death remains obscure. Therefore, we have tried to elucidate the mechanism of cytoprotective effect of (−)-DHMEQ against NO.

CHOP/GADD153 is considered to be a key modulator of NO-induced β-cell apoptosis [20]. Although the expression of CHOP mRNA was induced by NO, this induction was not prevented by (−)-DHMEQ (data not shown). Even though the precise apoptosis cascade downstream of CHOP remains to be clarified, several studies have indicated that prolonged ER stress or elevated CHOP expression causes ROS accumulation and leads to cell death [21,22]. In addition, it is known that $CHOP^{-/-}$ islet is resistant to NO-induced disruption of mitochondrial membrane potential [20], which is often associated with ROS accumulation. Moreover,

CHOP$^{-/-}$ mice are shown to display reduced levels of oxidative damage and promote β-cell survival [23]. These studies suggest that ROS may contribute to NO-induced β-cell death downstream of CHOP. Furthermore, ROS production is known as one of the contributing factors for NO-induced cell death in many cell types [24,25]. Thus, we speculated involvement of ROS in the NO-induced β-cell death. Indeed, we found that *N*-acetylcysteine (NAC), which possesses both direct and indirect antioxidant properties toward ROS, protected NO-induced cell death in Min6 cells (data not shown).

Nrf2 is a key regulator against oxidative stress in various cell types [10]. Recent studies have indicated that several chemopreventive agents repress NF-κB signaling with a concomitant activation of Nrf2 [13,14]. Since (−)-DHMEQ inhibited the endogenous NF-κB activation in Min6 cells (Fig. 1B), we next focused on Nrf2–ARE signaling pathway. As shown in Fig. 3A–D, (−)-DHMEQ induced transcriptional activation of Nrf2-target genes following the increase of Nrf2 protein levels and ARE luciferase reporter activity. In addition, NO-induced cell death was also prevented by treatment with a known Nrf2 activator tBHQ (Fig. 3E and F). Interestingly, although Nrf2 is known to be activated by nitrosative stress to counteract NO-induced apoptosis in several cell types [26,27], SNAP did not induce mRNA expression of Nrf2 downstream genes (Fig. 3D). This result may support the fact that β cell is one of the most susceptible cell type toward NO [28]. When the siRNA construct of Nrf2 was transfected in β cells, the cytoprotective effect of (−)-DHMEQ against NO was lowered in β cells (Fig. 4A). In addition, overexpression of Nrf2 prevented NO-induced β-cell death (Fig. 4B). These results indicate that (−)-DHMEQ-mediated Nrf2 activation is critical for the inhibition of NO-induced β-cell death. This finding is supported by the recent reports, in which Nrf2–ARE signaling would be essential for β-cell functions [29,30].

Even though the precise interaction between NF-κB and Nrf2 remains to be determined, evidence has been accumulating that there is a cross-talk between these two pathways. Liu et al. reported that NF-κB p65 subunit represses Nrf2–ARE pathway via two different mechanisms: (1) p65 deprives the transcriptional cofactor CREB-binding protein (CBP) from Nrf2. (2) p65 promotes recruitment of corepressor, histone deacetylase 3 (HDAC3), to the ARE. [31]. Moreover, Yu et al. reported that p65 interacts with Keap1, which leads to suppression of Nrf2–ARE pathway [32]. These two reports suggest the possible role of NF-κB in the negative regulation of Nrf2–ARE signaling. Furthermore, overexpression of IκB enhances ARE luciferase reporter activity with concomitant inhibition of NF-κB luciferase reporter activity [32]. Thus, it is likely that Nrf2 is negatively regulated by NF-κB.

In summary, we demonstrated that (−)-DHMEQ inhibited NO-induced β-cell death via activation of Nrf2–ARE signaling pathway. Since excessive NO plays a pivotal role in the progression of β-cell death in type 1 diabetes mellitus, Nrf2 appears to be a promising therapeutic target for prevention of β-cell dysfunction. This is the first study that clearly identifies a cytoprotective role of Nrf2 against NO in β cells. Since (−)-DHMEQ has various anti-inflammatory effects without any toxicity in animal models [33], (−)-DHMEQ may be a candidate for novel antidiabetic agent.

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