Biochemical and Biophysical Research Communications 387 (2009) 97–102

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dihydroavenanthramide D protects pancreatic β-cells from cytokine and streptozotocin toxicity

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ARTICLE INFO

Article history:
Received 24 June 2009
Available online 1 July 2009

Keywords:
Dihydroavenanthramide D
Cytokine
Streptozotocin
β-Cells
NF-κB

ABSTRACT

Dihydroavenanthramide D (DHAvD) is a synthetic analog to naturally occurring avenanthramide, which is the active component of oat. Although its anti-inflammatory, antiatherosclerotic, and antioxidant effects have been reported, the effect of DHAvD on type 1 diabetes is unknown. Therefore, in this study, the effect of DHAvD on cytokine- or streptozotocin-induced β-cell damage was investigated. Treatment of RINm5F insulinoma cells or isolated islets with IL-1β and IFN-γ induced β-cell damage through a NF-κB-dependent signaling pathway. DHAvD-pretreated RINm5F cells or islets showed resistance to cytokine toxicity, namely suppressed nitric oxide (NO) production, reduced the inducible form of NO synthase expression, and decreased β-cell destruction and the normal insulin secretion capacity. Furthermore, pretreatment with DHAvD blocked the development of type 1 diabetes in streptozotocin-treated mice. Prior injection with DHAvD maintained a normal range of plasma glucose and insulin, and retained immunoreactivity for insulin in the pancreas. These results suggest that DHAvD may be used to preserve functional β-cell mass.

Introduction

Type 1 diabetes is an autoimmune disease that is characterized at the cellular level by an augmented destruction of insulin-producing β-cells. Proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor-α, and interferon (IFN)-γ induce β-cell death *in vitro*, and the local release of the same cytokines by infiltrating lymphocytes and macrophages mediate β-cell destruction *in vivo* [1]. After binding to its receptor, IL-1β activates nuclear factor κB (NF-κB), which is considered to be a major regulator of iNOS expression that in turn leads to nitric oxide (NO) production [2]. NF-κB is initially located in the cytosol in an inactive form complexed with IκB, an inhibitory factor of NF-κB. IL-1β can cause dissociation of this complex, presumably by phosphorylation of IκB, resulting in the release of NF-κB. NF-κB then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription, including iNOS [3]. IFN-γ alone does not stimulate iNOS expression in β-cells, however it does prime them for IL-1β-induced iNOS expression [4].

Oats and oatmeal have been used for centuries to treat inflammatory diseases, arthritis, and skin afflictions such as redness, irritation, and itching. Dihydroavenanthramide D (DHAvD) is a synthetic analog to the naturally occurring avenanthramide, which is the active component in oats. Avenanthramide has been shown to be effective in reducing atherosclerosis [5], inflammation [6], itching [6,7], and oxidative stress [8]. A more recent study has shown that avenanthramide inhibits IL-1β-induced NF-κB in endothelial cells [9]. Due to the critical role of the NF-κB signaling pathway in type 1 diabetes development, in this study we have examined whether DHAvD could prevent cytokine or streptozotocin (STZ)-induced pancreatic β-cell damage using cultured RINm5F cells, isolated islets, and a STZ-induced diabetes animal model.

Materials and methods

Cell culture and reagents. Rat pancreatic β-cell line RINm5F cells were purchased from the American Type Culture Collection. DHAvD (SymCalmin®) was purchased from Symrise GmbH & Co. (Holzminden, Germany). IL-1β and IFN-γ were obtained from R&D Systems (Minneapolis, MN). All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Cell viability assay. The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan [10].

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doi:10.1016/j.bbrc.2009.06.133

Nitrite measurement. After treatment with cytokine for 24 h, 100 μl aliquots of the culture supernatants were incubated with 100 μl of a modified Griess reagent (1:1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 60% acetic acid) at room temperature for 5 min, at which time, the absorbance at 540 nm was measured using a spectrophotometer.

Whole cell and nuclear protein extracts. Cells were washed with PBS and lysed in CytoBuster™ Protein Extraction Buffer (Novagen, Madison, WI). The lysate was centrifuged at 10,000*g* for 5 min at 4 °C, and the supernatant was used as a whole cell protein extract. Cytoplasmic and nuclear extracts were prepared from cells using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).

Western blot analysis. RINm5F cells (5×10^6) or islets (30) were homogenized in 100 μl ice-cold lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Homogenates containing 20 μg protein were separated by SDS–PAGE, and the proteins were transferred to nitrocellulose membranes. The blot was probed with 1 μg/ml primary antibody against for iNOS, IκBα, p65, p50, β-actin, and PCNA (Santa Cruz Biochemicals, Santa Cruz, CA). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA) was used as a secondary antibody.

Electrophoretic mobility shift assay (EMSA). NF-κB activation was assayed by a gel mobility shift assay using nuclear extracts from control and treated cells. An oligonucleotide containing the κ-chain binding site (κB, 5′-CCGGTTAACAGAGGGGCTTTCCGAG-3′) was synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [α-^{32}P]dCTP. Binding reactions containing labeled oligonucleotide (10,000 cpm), 10 μg nuclear extract protein, and binding buffer (10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI · dC), and 1 mM dithiothreitol) in a final volume of 20 μl were incubated for 30 min at room temperature. Reaction mixtures were separated by electrophoresis on 4% polyacrylamide gels in 0.5× Tris–borate buffer, then the gels were dried and visualized by autoradiography. The specificity of the DNA–protein interaction for NF-κB was demonstrated using a competition assay with a 50-fold excess of unlabeled oligonucleotide.

Type 1 diabetes induction. Specific pathogen-free male ICR mice were purchased from Orientbio Inc. (Seoungnam, Korea). To induce diabetes, mice were injected via the tail vein with 80 mg STZ/kg of body weight dissolved in 0.1 M sodium citrate buffer (pH 4.0). Mice received intraperitoneal injections of 1.5 g/kg DHAvD daily for 3 days before the administration of STZ. Plasma glucose was assayed by the glucose oxidase method (Sigma), and plasma insulin was measured using a radioimmunoassay kit (Linco Research, St. Charles, MO). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Chonbuk National University.

Glucose-stimulated insulin secretion assay. Pancreatic islets were isolated from male Sprague–Dawley rats using the collagenase digestion method. Islets were incubated for 24 h with IL-1β (1 U/ml) and IFN-γ (100 U/ml) in the presence or absence of DHAvD. The islets were then washed three times in Krebs–Ringer bicarbonate buffer (25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 0.1% bovine serum albumin, pH 7.4) containing 3 mM D-glucose. Insulin secretion assays were performed in the presence of either 5.5 or 20 mM D-glucose.

Immunohistochemistry. Immunohistochemical staining was performed with the DAKO Envision system (DAKO, Carpinteria, CA). Pancreases were removed and immediately placed in fixative (10% formalin solution in 0.1 M PBS). Histological sections of 4 μm thickness were cut from formalin-fixed paraffin-embedded tissue blocks. After deparaffinization, tissue sections were treated using a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer. After blocking endogenous peroxidase, sections were incubated with Protein Block Serum-Free (DAKO) to block nonspecific staining, and then incubated with anti-insulin antibodies (Santa Cruz Biochemicals). Peroxidase activity was detected using the enzyme substrate 3-amino-9-ethyl carbazole.

Statistical analysis. Statistical analyses of the data were performed using the ANOVA and Duncan's tests. Differences with a p-value < 0.05 were considered statistically significant.

Results

Protective effect of DHAvD on cytokine-induced RINm5F cell death

RINm5F cells were pretreated with or without DHAvD for 3 h before exposure to IL-1β (1 U/ml) and IFN-γ (100 U/ml) for 48 h, then they were harvested and their viability assessed using an MTT assay. Cytokine treatment significantly reduced cell viability to 55.2 ± 0.9% compared to the control (Fig. 1A). However, pretreat-

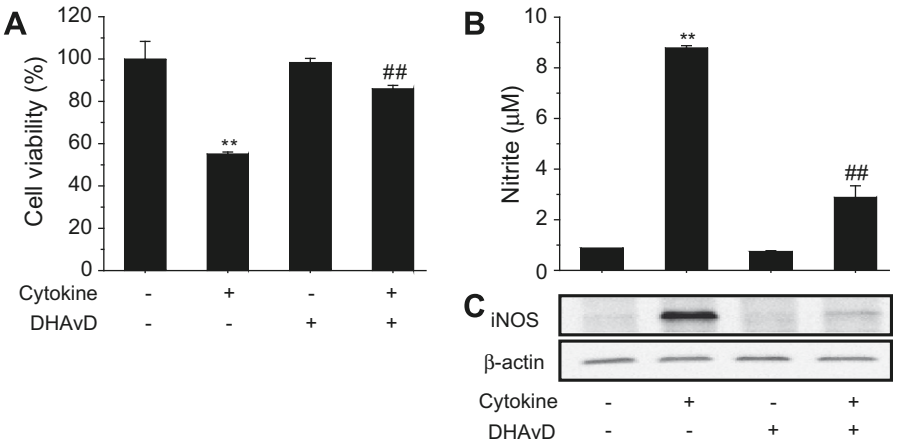

Fig. 1. DHAvD protected against cytokine-induced cell death and NO production and iNOS expression in RINm5F cells. RINm5F cells (1×10^5) were pretreated with DHAvD (5 μM) for 3 h, then IL-1β (1 U/ml) and IFN-γ (100 U/ml) were added for 48 h. Cell viability was determined using the MTT assay (A). RINm5F cells were treated with IL-1β (1 U/ml) and IFN-γ (100 U/ml) with or without a 3 h DHAvD pretreatment (5 μM). Following 24 h of incubation, NO production and iNOS protein expression were determined (B). Each value represents the mean ± SE of three independent experiments. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < 0.01$ vs. cytokine.

ment with 5 μM DHAvD increased viability to 85.9 ± 1.6%, compared with control cells.

It has been reported that IL-1β and IFN-γ-mediated destruction of β-cells is caused by the increased production of NO [10,11]. Incubation of RINm5F cells with cytokines for 24 h resulted in significant production of nitrite (a stable oxidized product of NO). However, pretreatment with DHAvD significantly decreased the cytokine-mediated production of nitrite (Fig. 1B), and this reduction was well correlated with the lowered cytotoxicity. To determine whether DHAvD inhibited NO production via the suppression of iNOS expression, changes in the expression of iNOS protein was investigated by Western blot analysis. iNOS protein expression was markedly increased in cells treated with cytokines, and suppressed in cells pretreated with DHAvD (Fig. 1B). These results indicate that the cytoprotective effect of DHAvD against IL-1β and IFN-γ results from the suppression of NO production.

Effect of DHAvD on cytokine-induced activation of NF-κB in RINm5F cells

NF-κB has been implicated in the transcriptional regulation of cytokine-induced expression of iNOS. Therefore, we studied the effect of DHAvD on cytokine-stimulated translocation of NF-κB from the cytoplasmic compartment to the nucleus, and DNA binding in RINm5F cells. Cytokine-treated cells showed an increased binding activity to a NF-κB consensus sequence (Fig. 2A), and increased levels of p65 and p50 subunits in their nuclei (Fig. 2B), compared with unstimulated cells. However, cytokine-induced activation of NF-κB was markedly suppressed by pretreatment with DHAvD, suggesting that DHAvD inhibits iNOS expression through the inhibition of NF-κB activation.

We previously reported that IκBα, but not IκBβ, is the major participant in IL-1β and IFN-γ-induced NF-κB activation [10]. Therefore, we investigated IκBα protein levels in the cytoplasmic fraction after cytokine treatment. Cytokine-treated RINm5F cells showed increased degradation of IκBα protein in the cytoplasm when compared to a similar fraction from unstimulated cells; pretreatment with DHAvD inhibited this cytokine-induced IκBα degradation (Fig. 2B).

Effect of DHAvD on cytokine-induced activation of the NF-κB and glucose-stimulated insulin secretion in rat islets

We next assessed the preventive effects of DHAvD using isolated islets from rat. Incubation of islets with cytokines for 1 h increased NF-κB signal pathway activity, namely, the increased DNA binding, nuclear translocation of NF-κB subunits, and increased cytoplasmic degradation of IκBα (Fig. 3A). In addition, production of NO and expression of iNOS proteins were markedly increased by cytokine treatment (Fig. 3B). Similar to the results obtained in RINm5F cells, pretreatment of islets with DHAvD abolished the effects of the cytokine and resulted in suppressed NF-κB activity, as well as similar levels of NO production and iNOS expression as those of the controls. To add functional data, we also measured insulin secretion in response to treatment with 20 mM glucose. Control islets secreted insulin at a concentration of 3.4 ± 0.7 ng/ml, whereas insulin secretion from cytokine-treated islets decreased significantly to 0.8 ± 0.1 ng/ml ($p < 0.01$) (Fig. 3C). Pretreatment with DHAvD blocked the effect of the cytokines and maintained islet cell insulin secretion to levels similar to those in the controls.

Effect of DHAvD on the STZ-induced type 1 diabetes model

To assess the potential of DHAvD to protect against STZ-mediated type 1 diabetes, ICR mice were injected daily with 1.5 g/kg DHAvD for 3 days, and then injected with a single high dose of STZ (80 mg/kg). Levels of fasting glucose at day 5 were significantly higher in the STZ-treated group than in the control group (Fig. 4A). Conversely, serum insulin level was significantly lower in the STZ group (Fig. 4B). Histological analysis at day 6 revealed degenerative and necrotic changes in islet cells from mice treated with STZ alone, which is consistent with the results described above (Fig. 4C). Furthermore, immunohistochemical staining for insulin clearly showed fewer insulin-immunoreactive β-cells in the STZ-treated group than in the control group. However, prior injection with DHAvD blocked the STZ-induced islet destruction and maintained the number of islet cells that secreted insulin to the control level. To examine whether DHAvD treatment also affects NF-κB

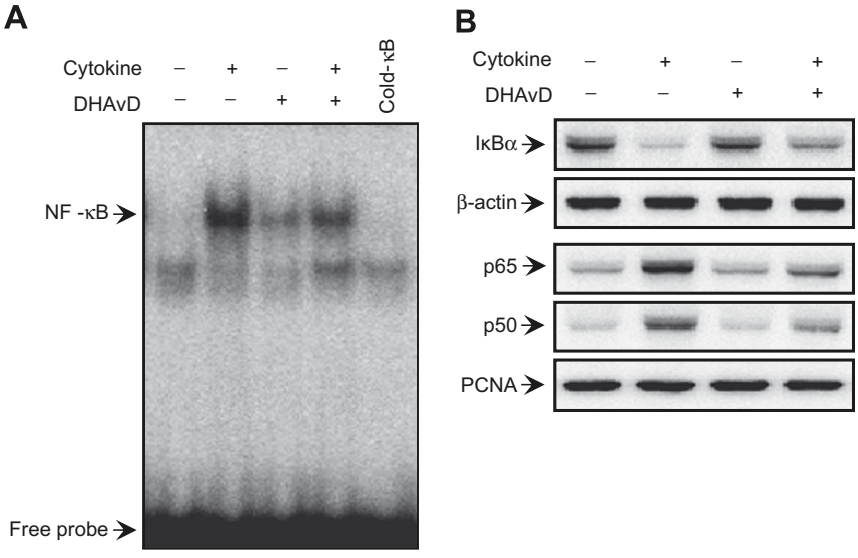

Fig. 2. DHAvD inhibited the cytokine-induced NF-κB activation pathway. RINm5F cells (5×10^6) were treated with IL-1β (1 U/ml) and IFN-γ (100 U/ml) with or without a 3 h DHAvD pretreatment (5 μM). Following 30 min of incubation, the DNA-binding activity of NF-κB was analyzed by EMSA (A). Protein levels of p50 and p65 in the nuclear fractions and IκBα in the cytosol extracts were determined by Western blotting (B). β-Actin and PCNA were used as loading controls for cytosolic and nuclear proteins, respectively. EMSA and Western blotting data are representative of three separate experiments.

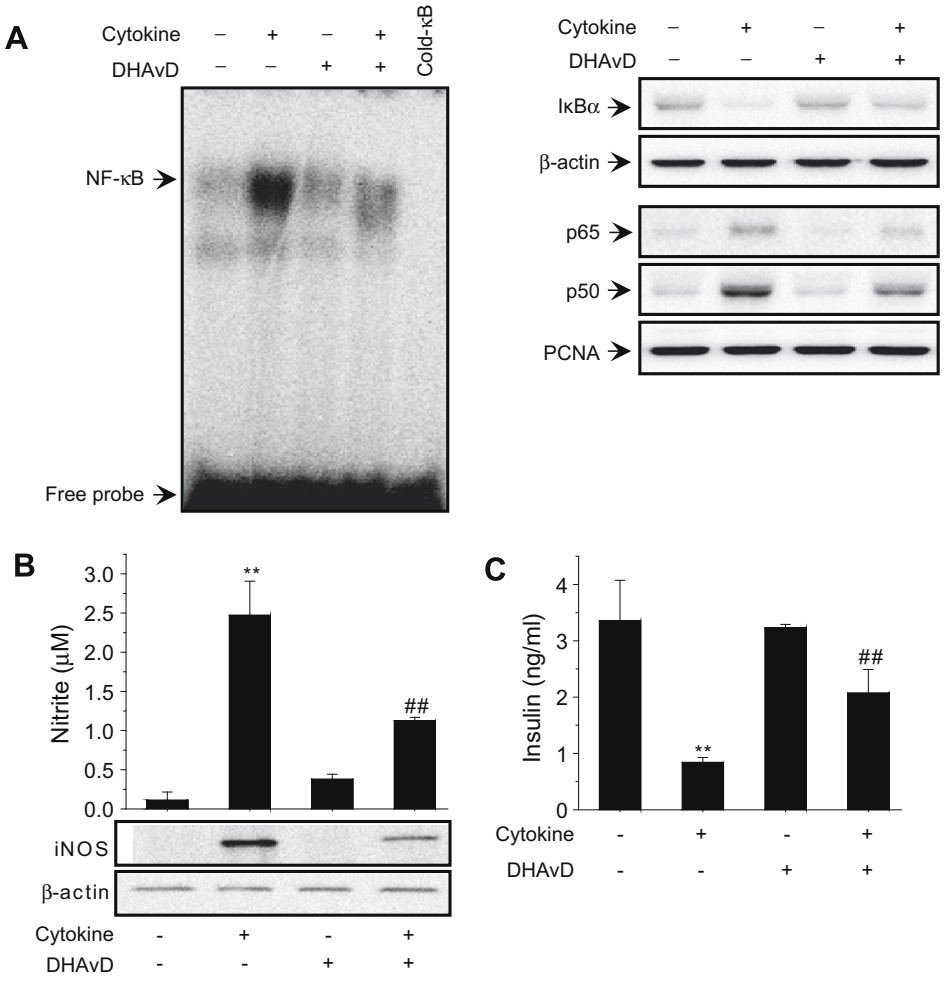

Fig. 3. DHAvD inhibited the cytokine-induced activation of the NF-κB pathway and maintained normal glucose-stimulated insulin secretion in rat islets. Rat islets (300 total) were treated with IL-1β and IFN-γ with or without a 3 h DHAvD pretreatment (5 μM). The DNA binding of NF-κB, nuclear transclocation of NF-κB subunits, and cytoplasmic degradation of IκBα were determined after 1 h (A), and nitrite production and iNOS protein expression were determined after 24 h (B). Rat islets (10 islets/500 μl) were treated with IL-1β and IFN-γ with or without a 3 h DHAvD pretreatment. After 24 h incubation, glucose-stimulated insulin secretion was quantified (C). The results of three independent experiments are expressed as the mean ± SE. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < 0.01$ vs. cytokine.

signaling *in vivo*, nuclear extracts prepared 30 min after STZ treatment were analyzed by EMSA. STZ treatment rapidly increased the level of NF-κB DNA binding, nuclear translocation of NF-κB subunits, and cytoplasmic degradation of IκBα (Fig. 4D), whereas pretreatment with DHAvD prior to injection with STZ completely inhibited NF-κB activation, suggesting that NF-κB activation may be a critical determinant in the STZ-induced islet destruction model.

Discussion

In the present study, we demonstrated for the first time that pretreatment with DHAvD effectively protected pancreatic β-cells against cytokine toxicity. We also demonstrated that intraperitoneal administration of DHAvD prevented STZ-induced type 1 diabetogenesis. Inhibition of the NF-κB signal pathway is a contributory factor in DHAvD-induced β-cell rescue.

We first showed that DHAvD exerted a cytoprotective effect against cytokine toxicity in RINm5F cells and isolated rat islets. The β-cell destruction and subsequent insulin deficiency observed in type 1 diabetes is at least partially believed to be mediated by cytokines. A number of genes are induced by cytokines and their expressions are dependent on the two transcription factors, NF-κB and STAT. Pretreatment with DHAvD suppressed the cytokine-induced NF-κB signaling pathway: IκBα degradation, nuclear translocation of p50 and p65 subunits, and DNA binding of NF-κB. Since iNOS is a downstream target of NF-κB, it is not surprising that DHAvD-pretreatment blocks cytokine-induced NO production. Our results are in agreement with those from two recent studies showing that DHAvD inhibits NF-κB pathway in keratinocytes [6] and endothelial cells [9]. These investigators demonstrated that avenanthramide or synthetic avenanthramide exhibit an anti-inflammatory effect by suppressing NF-κB activation. In contrast, Nie et al. [12] has reported that avenanthramide-2c enhances eNOS mRNA expression and NO production in smooth muscle cells and human aortic endothelial cells. Our results and the above reports suggest that avenanthramide enhances or suppresses NO production according to the target cells or avenanthramide used. We also observed that cytokine-induced JAK-STAT phosphorylation was partially inhibited by DHAvD (data not shown). As we have reported, interruption of the JAK-STAT pathway is an effective way to protect against the deleterious effects of cytokines on β-cells [13–15]. Even though the data and mechanism by which the JAK-STAT pathway is inhibited by DHAvD are not addressed in the present study, we believe DHAvD has a protective effect against cytokines through suppressing both NF-κB and JAK-STAT pathways.

Pancreatic β-cells are considered exceptionally vulnerable to the cytotoxic actions of reactive oxygen species (ROS) because of

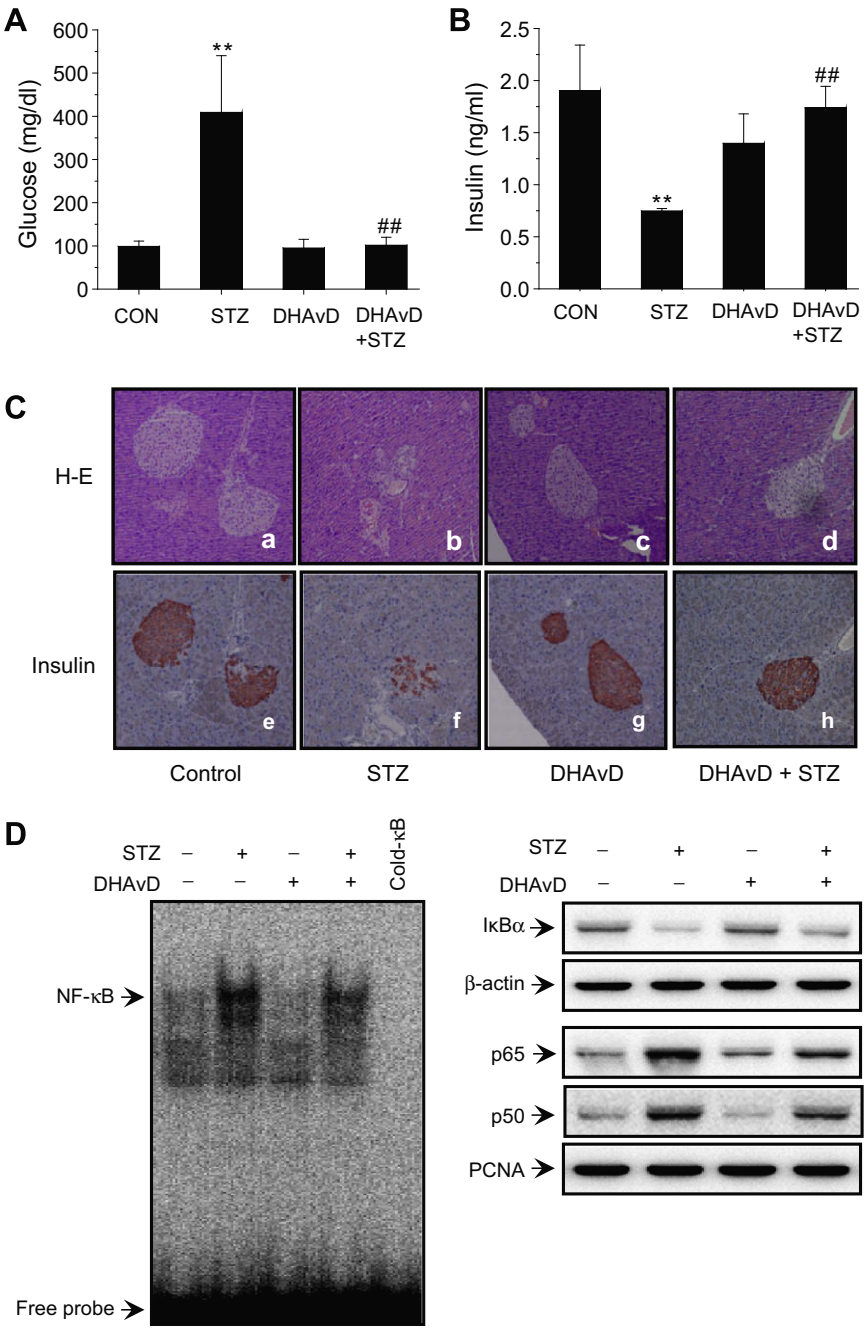

Fig. 4. DHAvD protected islets from STZ-induced destruction. Type 1 diabetes was induced as described in Materials and Methods. Levels of fasting glucose (A) and insulin (B) were determined. (C) Pancreases were obtained from normal controls (a,e), and from mice injected with STZ (b,f), DHAvD (c,g), or DHAvD and STZ (d,h). The cellular morphologies of these islets and adjoining exocrine regions were stained with H–E (a–d). Sections were labeled with insulin antibodies and peroxidase-labeled anti-rabbit IgG, and then examined by microscopy (e–h). (D) Nuclear extracts were prepared 30 min after STZ injection and the DNA binding of NF-κB, nuclear translocation of p65 and p50, and cytoplasmic degradation of IκBα were analyzed. The results are expressed as the mean ± SE. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < 0.01$ vs. STZ-injected group.

their relatively low levels of antioxidant enzymes [16]. Experimental studies have shown that ROS are produced in cytokine-stimulated [17,18] pancreatic β-cells, and that transgenic overexpression of antioxidant enzymes protects β-cells from cytokine toxicity [19]. Recently, we have also reported that Nrf2 activation by sulforaphane treatment or by genetic overexpression blocks H_2O_2 production, NF-κB activation, and cytokine toxicity [20]. Furthermore, ROS induce NF-κB activation [21], suggesting that ROS play a role in the pathway by which cytokines activate NF-κB. A recent study by Chen et al. [8] shows that avenanthramide is bioavailable in humans and has antioxidant activity. Therefore, the cytoprotective effect of DHAvD observed in this study may be due to the suppression of the NF-κB signaling pathway at the level of ROS production. Additional studies are required to establish whether DHAvD affects ROS production in pancreatic β-cells.

Protective effects of DHAvD were also observed in animal studies. Intraperitoneal injection of DHAvD prior to STZ treatment protected against pancreatic islet damage in mice. STZ destroys islet cells by several mechanisms, including the production of ROS during its metabolism [22], the activation of pancreatic NF-κB [23], and the induction of pronounced immune and inflammatory responses [24]. Following injection with STZ, pancreatic islets showed obvious β-cell damage with degenerative and necrotic changes, and reduced staining for insulin. We also observed in-

creased NF-κB activation in the pancreas of STZ-treated mice, and that the ability of DHAvD to protect against the development of type 1 diabetes was correlated with its ability to inhibit NF-κB activation. This protective effect of DHAvD was paralleled by normalized glucose and insulin levels, and preserved functional β-cell masses. Therefore, it seems likely that the DHAvD-induced suppression of the NF-κB pathway in STZ-treated mice is crucial for its protective role.

Several pathways (e.g. oxidative stress, ER stress, glucotoxicity, or lipotoxicity) are known to destroy β-cells via activating the NF-κB pathway. Hypothetically, this gives us only one approach, the ability to block NF-κB signaling, to save β-cell mass and thereby prevent diabetes development. Unsurprisingly, NF-κB has been extensively studied to determine its role in the pathogenesis of diabetes, and NF-κB inhibition has been explored as a therapeutic approach for the disease. We also have reported that regulation of NF-κB by SIRT1 protects β-cells from cytokine toxicity [25]. In the present study, we presented evidence that DHAvD treatment prevented NF-κB activation in cytokine-treated β-cells, which in turn inhibited cell damage. Intraperitoneal injection of DHAvD also maintained insulin secretion capacity in the STZ-induced diabetes model.

Acknowledgments

This work was supported by the Ministry of Science & Technology (MoST)/Korea Science & Engineering Foundation (KOSEF) through the Diabetes Research Center at Chonbuk National University (R13-2008-005-0000-0).

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