Exendin-4 inhibits interleukin-1β-induced iNOS expression at the protein level, but not at the transcriptional and posttranscriptional levels, in RINm5F β-cells

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Abstract

Cytokines such as interleukin-1β (IL-1β) stimulate inducible nitric oxide synthase (iNOS) expression and nitric oxide overproduction leading to β-cell damage. Meanwhile, glucagon-like peptide-1 (GLP-1) and its potent analog exendin-4 (EX-4) were well known for β-cell proliferation. However, the protective mechanisms of GLP-1 in β-cells exposed to cytokines were not fully elucidated. Therefore, the effects of EX-4 on the IL-1β-induced iNOS gene expression were investigated employing RINm5F β-cells. EX-4 inhibited IL-1β-induced iNOS protein expression and nitrite production. However, northern blot and promoter analyses showed that EX-4 failed to inhibit IL-1β-induced iNOS mRNA expression and iNOS promoter activity. By electrophoretic mobility shift assay (EMSA), EX-4 did not alter the binding activity of NF-κB to the iNOS promoter. Consistent with the EMSA result, EX-4 did not inhibit nuclear translocation of p65. We also tested the effect of EX-4 on iNOS mRNA stability. Actinomycin D chase experiments showed that EX-4 did not affect the decay rate of iNOS mRNA and the promoter assay using the construct containing 3′-untranslated region of iNOS showed that EX-4 did not alter the stability of iNOS mRNA. Meanwhile, forskolin significantly inhibited IL-1β-induced iNOS protein, which was reversed by H-89, a protein kinase A (PKA) inhibitor. Moreover, EX-4 pretreatment restored IL-1β-induced decrease in cAMP toward control level. Additionally, the cycloheximide chase study demonstrated that EX-4 significantly accelerated iNOS protein degradation. We therefore concluded that EX-4 inhibited IL-1β-induced iNOS protein and nitrite production via cAMP/PKA system irrespective of both transcriptional and posttranscriptional mechanisms of iNOS gene, and this inhibitory effect of EX-4 appears to be regulated at posttranslational level.
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Introduction

The main feature of type 1 diabetes mellitus is an autoimmune insulitis, that is, the infiltration of inflammatory cells around the pancreatic islets (Andre *et al.* 1996). The insulitis is primarily mediated by proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ; Mandrup-Poulsen 2001). Specifically, IL-1β induces the expression of inducible nitric oxide synthase (iNOS) in islet β-cells leading to the overproduction of nitric oxide (NO) that mediates cytotoxicity of β-cells (Southern *et al.* 1990, Rabinovitch 1998).

Accumulating evidence shows that β-cells are the primary source of cytokine-induced free radical formation (Salehi *et al.* 1996, Tabatabaie *et al.* 2003). In particular, low antioxidant defense systems of β-cells are considered to be a target of free radicals, leading to β-cell dysfunction and type 1 diabetes mellitus (Ho & Bray 1999). Some previous studies indicate that NO scavengers or reduction of iNOS enzyme activity have been implicated in the protection of β-cells exposed to cytokines (Corbett & McDaniel 1992, Mandrup-Poulsen 1996). In addition, though inflammatory cytokines IL-1β, TNF-α, and INF-γ signal via different receptors, these combined cytokines induce numerous target genes including iNOS. Among these genes, almost 50% are NO-dependent, which suggests a pivotal role of iNOS in cytokine-mediated β-cell injury (Kutlu *et al.* 2003). Therefore, the approaches to inhibit or reduce iNOS expression may be necessary for the prevention or inhibition of β-cell damage.

Glucagon-like peptide-1 (GLP-1) and its potent agonist exendin-4 (EX-4) have received great attention because of their insulinotropic and β-cell-proliferating effects (Ørskov 1992, Egan *et al.* 2003, Bulotta *et al.* 2004, Baggio & Drucker 2007). Both GLP-1 and EX-4 activate multiple signaling pathways such as cAMP/protein kinase A (PKA) system,

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phosphatidylinositol 3-kinase, and mitogen-activated protein kinase (MAPK), which lead to β-cell maintenance and growth (Jhala *et al.* 2003, List & Habener 2004, Stoffers 2004). In addition, GLP-1 induces immediate early response genes such as c-fos, c-jun, and early growth response-1 (Egr-1), which are involved in β-cell growth and differentiation (Susini *et al.* 1998, Stoffers 2004). Recently, we found that EX-4 induces transcriptional activation of cyclin D1 and Egr-1 genes, which were known to be involved in cell proliferation, in INS-1 β-cells (Kang *et al.* 2006, 2007, Kim *et al.* 2006, 2008).

Despite diverse effects of GLP-1, its protective mechanisms on cytokine-induced iNOS expression are incompletely understood. GLP-1 and glucagon reduced NO production through the cAMP level increase, in high glucose- and IL-1β-stimulated islets respectively (Belin *et al.* 1999, Jimenez-Feltstrom *et al.* 2005). GLP-1 suppressed excessive NO generation and iNOS activity in diabetic rat islet via the activation of cAMP/PKA system (Salehi *et al.* 2008). EX-4 decreased cytokine-induced iNOS protein expression through protein kinase B activation in INS-1 cells (Li *et al.* 2005). However, these studies were mainly limited at the levels of iNOS protein expression and enzyme activity. Therefore, this study was aimed to explore the inhibitory effects of EX-4 on IL-1β-induced iNOS expression focusing on iNOS gene transcription mechanism.

Materials and Methods

Materials

RINm5F (CRL-11605) cells, a rat insulinoma cell line, were obtained from American Type Tissue Collection (Manassas, VA, USA). FBS, RPMI 1640 medium, and OPTI-MEM were purchased from Gibco BRL. EX-4 was from Bachem AG (Torrance, CA, USA). Recombinant human IL-1β and Cyclic AMP Assay kit were from R&D systems (Minneapolis, MN, USA). Forskolin, PD98059, SB203580, H-89, and cycloheximide were from Calbiochem (La Jolla, CA, USA). AccuPrep genomic DNA extraction kit was from Bioneer (Daejon, Korea). Top-Pfu DNA polymerase was from Bio-Online (Seoul, Korea). Lipofectamine 2000 reagent was from Invitrogen. BCA protein assay kit was from Perbio Science (Erembodegem, Belgium). Griess Reagent System, pGL3 Luciferase Reporter Vector, pSV-β-gal, pGEM-T Easy Vector, and T4 polynucleotide kinase were from Promega. Anti-mouse iNOS antibody was from BD Transduction Laboratories (Palo Alto, CA, USA). Western blotting Luminol reagent, NF-κB oligonucleotides probe, and anti-p65 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RNA STAT-60 was from TEL-TEST (Friendswood, TX, USA). First Strand cDNA Synthesis Kit, alkaline phosphatase-conjugated anti-dogoxigenin (DIG) antibody, and CDP-Star was from Roche Diagnostics. All other reagents were purchased from Sigma. EX-4 was prepared in RPMI 1640 containing 0·1% BSA to prevent the peptide from adherence to plastic surfaces. Forskolin was dissolved in ethanol at 100 mM stock solution. PD98059, SB203580, and H-89 were dissolved in DMSO at 100 mM stock solution and added to the culture medium. The final concentration of vehicles did not exceed 0·1%.

Drugs treatment

RINm5F cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), and maintained at 37 °C in humidified air containing 5% CO_2. Cells were subcultured weekly and passages 19–30 were used exclusively. We fixed the concentration (100 pg/ml) and incubation time of IL-1β based on our previous study (Kim *et al.* 2004). The cells were plated at a concentration of 2×10^5/ml except for transient transfection experiment. At 70% confluency, the cells were incubated with serum-free RPMI medium for 16 h, and then pretreated with EX-4 (1 or 10 nM) and forskolin (10 μM) for 30 or 60 min, and next incubated with IL-1β (100 pg/ml) for indicated times. Pharmacological inhibitors (H-89 (5 μM): a PKA inhibitor; PD98059 (10 μM): an MEK inhibitor; SB203580 (10 μM): p38 MAPK inhibitor) were added 30 min prior to stimulation of cells with IL-1β and EX-4.

Measurement of NO as nitrite

RINm5F cells were treated with EX (1 and 10 nM) for 1 h, and then incubated with IL-1β (100 pg/ml) for an additional 24 h. Released nitrite, a stable product of NO in aqueous medium, was measured using Griess Reagent System as described previously (Kim *et al.* 2004). Briefly, the culture medium was mixed with an equal volume of sulfanilamide solution (1% in 5% phosphoric acid) and of *N*-1-naphtylethylenediamine dihydrochloride solution (0·1% in water). The absorbance was measured at 540 nm on MR700 Microplate Reader (Dynatech Laboratories Inc., Chantilly, VA, USA). Nitrite concentrations were then determined from a calibration curve of standard $NaNO_2$ concentrations against absorbance. Data are expressed as mM per mg protein to adjust for the differences in the cell mass among the groups.

Western blot analysis

RINm5F cells were treated with EX-4 (1 and 10 nM) or *N*-nitro-L-arginine methylester (L-NAME, 1 mM) for 1 h, and then incubated with IL-1β (100 pg/ml) for an additional 16 h. Western blot analysis was performed essentially as described previously (Kim *et al.* 2004, 2006). Briefly, the cells were harvested, and solubilized with RIPA buffer (25 mM Tris–HCl, pH 7·4, 0·1% SDS, 0·1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM

Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). The soluble fraction was collected and protein content was determined by BCA. Thirty micrograms total protein was separated on an 8% SDS-PAGE and transferred onto nitrocellulose membrane. After blocking with 5% skimmed milk, the membrane was incubated with anti-mouse iNOS IgG (0·5 μg/ml of TTBS). Then, the membrane was probed with peroxidase-conjugated anti-mouse IgG (0·5 μg/ml TTBS). The signal was visualized by enhanced chemiluminescence system. For the nuclear translocation of p65 protein, an NF-κB subunit, the cells were pretreated with EX-4 for 30 min, then incubated with IL-1β for 30 min. Nuclear and cytosolic fractions were obtained using sequential hypotonic and hypertonic solutions. Equal loading and transfer of samples were verified by Ponseau S staining or the band intensity of β-tubulin (52 kDa).

Northern blot analysis

RINm5F cells were treated with EX-4 (1 and 10 nM) or NMMA (100 μM) for 1 h, and then incubated with IL-1β (100 pg/ml) for an additional 6 h. Total RNA isolation and the method for northern blot analysis were described previously (Kang *et al.* 2006, Kim *et al.* 2008). The iNOS probe was demonstrated in the previous study (Kim *et al.* 2004). Equal loading of sample was verified by GAPDH or the ribosomal 18S bands.

Measurement of cellular cAMP concentration

RINm5F cells were cultured in 6-well culture plates at a concentration of 1×10^6 cells/well. The cells were incubated with EX-4 (10 nM) for 30 min, and then incubated with IL-1β (100 pg/ml) for 30 min. The phosphodiesterase inhibitor 3-iosbutyl-1-methylxanthine (IBMX, 250 μM) were added to prevent cAMP breakdown. The cellular cAMP content was measured using cAMP assay kit according to the manufacturer's instructions. The absorbance was measured at 450 nm on MR700 Microplate Reader (Dynatech Laboratories Inc.).

Plasmids

Rat iNOS promoter ranging from −1173 to +33 was generated by PCR amplification method and cloned into pGL3 basic vector, then designated as piNOS-LUC (Kim *et al.* 2004). To examine the effect of the 3′-untranslated region (UTR) on iNOS mRNA stability, cDNA for 3′-UTR of iNOS mRNA was amplified with the primers (GenBank/EMBL accession number: U03699, forward primer: 5′-GCTCTAGACACCCAGAAGAGT-TACAGCATC with XbaI (underlined), reverse primer: 5′-GCTCTAGACTTGATCAAACACTCATTTTATTAA-AATAAAATG with XbaI (underlined)). This resultant cDNA was inserted into piNOS-LUC to generate piNOS-UTR. The construct was verified by sequencing using An ABI PRISM 310 genetic analyzer (PerkinElmer Ltd Co., Seoul, Korea).

Transient transfection and luciferase reporter assay

Transient transfection was performed by lipofection using Lipofectamine 2000 reagent as described previously (Kim *et al.* 2006). Briefly, RINm5F cells were plated at a density of 4×10^5/well in a 6-well plate 4 days before the transfection. Cells at 70% confluence were cotransfected

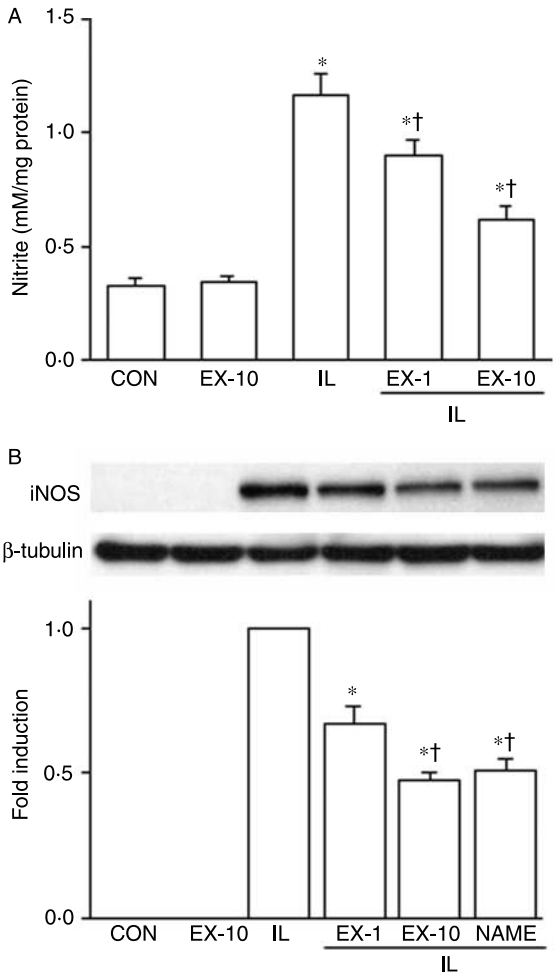

Figure 1 The inhibitory effects of exendin-4 on IL-1β-induced nitrite formation and iNOS protein expression in RINm5F cells. (A) The cells were pretreated with indicated doses of exendin-4 (EX, 1 and 10 nM) for 1 h, and then incubated with IL (100 pg/ml) for an additional 24 h. Nitrite production was measured in the same way mentioned above. Data are expressed as mean±S.D. ($n=4$). *$P<0·05$ versus CON; †$P<0·05$ versus IL. (B) Following the pretreatment of EX (1 and 10 nM) or L-NAME (1 mM) for 1 h, the cells were incubated with IL (100 pg/ml) for an additional 16 h. Western blot analysis for iNOS was performed. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean (±S.D.) of relative band density ($n=3$). *$P<0·05$ versus IL; †$P<0·05$ versus EX+IL.

with piNOS-LUC or piNOS-UTR (1 μg), and pSV-β-gal (0·1 μg). Following the serum starvation for 24 h, the cells were treated with EX-4 (10 nM) or forskolin (10 μM) for 30 min, and then incubated with IL-1β (100 pg/ml) for an additional 10 h. Luciferase and β-galactosidase activities were measured with a luminometer (TD20/20, Turner Designs Instrument, Sunnyvale, CA, USA) and MR700 Microplate Reader (Dynatech Laboratories Inc.) respectively. Transfection efficiencies were normalized by a ratio of luciferase activity to β-galactosidase activity obtained from the same sample.

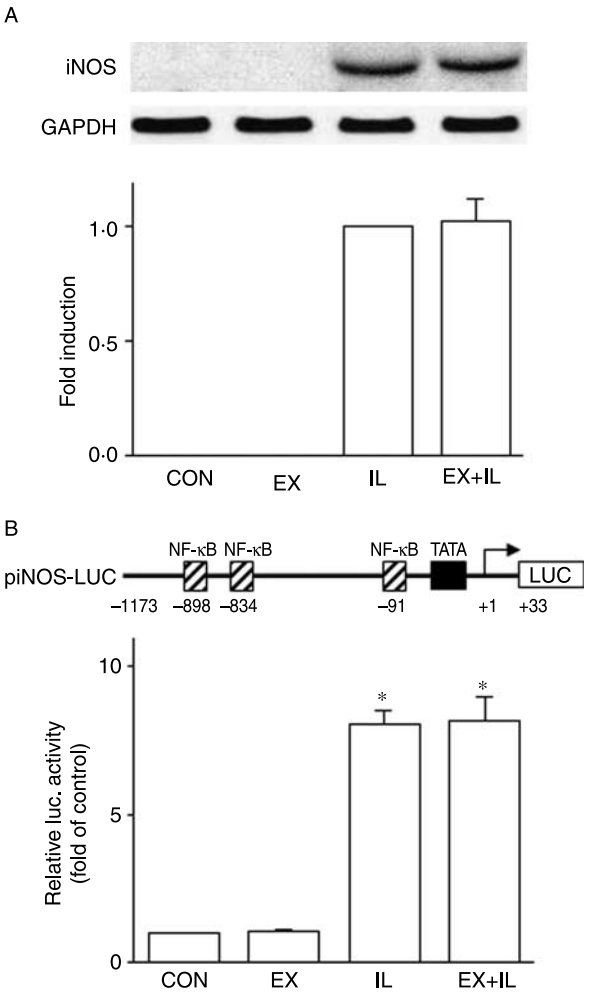

Figure 2 The effects of exendin-4 on IL-1β-induced iNOS mRNA expression and iNOS promoter activity in RINm5F cells. (A) The cells were pretreated with exendin-4 (EX, 10 nM) for 1 h, and then incubated with IL-1β (IL, 100 pg/ml) for an additional 6 h. The expressions of iNOS mRNA were measured by northern blot analysis described in materials and methods. Equal loading of sample (10 μg) was verified by the GAPDH. Data are expressed as mean (±S.D.) of relative band density ($n=3$). (B) Schematic representation of the rat iNOS promoter gene focusing on NF-κB binding site. The cells were transiently cotransfected with piNOS-LUC (1 μg) and pSV-β-gal (0·1 μg, as an internal control). Following the serum starvation, the cells were pretreated with EX (10 nM) for 30 min, then cultured for an additional 10 h with IL (100 pg/ml). Cells were then solubilized in lysis buffer and luciferase and β-galactosidase activities were measured. Data are expressed as mean±S.D. ($n=4$) and each value is expressed relative to the control value. *$P<0·05$ versus CON. CON, control without any treatment; LUC, luciferase.

Electrophoretic mobility shift assay

RINm5F cells were treated with EX-4 for 30 min, and then incubated with IL-1β (100 pg/ml) for 30 min. Electrophoretic mobility shift assay (EMSA) was performed to examine the binding activity of nuclear proteins to NF-κB binding sites of rat iNOS promoter by the method reported previously (Kim *et al.* 2004, 2006). For the DNA binding activity of NF-κB, the oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing κB binding sites (underlined) was used. The antibody against p65, an NF-κB subunit, was used for immune-supershift assay.

iNOS mRNA stability assay

RINm5F cells were treated with EX-4 (10 nM) for 1 h, and then incubated with IL-1β (100 pg/ml) for 6 h, and next exposed to actinomycin D (0·5 μg/ml) to block RNA synthesis. The cells were harvested at 0, 1, 2, 4, and 6 h after the addition of actinomycin D. Total RNA was extracted and northern blot analysis for iNOS mRNA was performed.

iNOS protein stability study

RINm5F cells were incubated with IL-1β (100 pg/ml) for 16 h, and then exposed to cycloheximide (2 μg/ml) for 1 h to stop protein synthesis, and next treated with EX-4 (10 nM). The cells were harvested at 0, 1, 3, and 6 h after the addition of EX-4. Total protein was extracted and western blot analysis for iNOS protein was performed.

Statistical analysis

The relative band densities were quantified using Scion Imaging software (Scion Corporation, Frederick, MD, USA). All data obtained from each experiment were expressed as mean±S.D. The data were analyzed using one-way ANOVA with Origin 7·0 software (Microcal Software, Northampton, MA, USA). Statistical comparisons among the groups were done by Bonferroni's multiple range *t*-test after the ANOVA. $P<0·05$ was accepted as statistically significant.

Results

Effects of EX-4 on IL-1β-induced NO production and iNOS protein

We previously evaluated the optimal conditions to observe the effect of IL-1β on nitrite production in RINm5F cells. IL-1β significantly induced nitrite production at a

concentration 100 pg/ml or above (Kim *et al.* 2004). The effect of EX-4 on IL-1β-induced NO production were examined. EX-4 significantly inhibited IL-1β-induced NO production (Fig. 1A). In inhibiting iNOS protein expression, EX-4 showed as much propensity as NMMA, an NOS inhibitor in RINm5F cells (Fig. 1B).

Effects of EX-4 on IL-1β-induced iNOS mRNA and promoter activity

To determine whether the effect of EX-4 on IL-1β-induced iNOS protein expression could have resulted from the inhibition of iNOS gene transcription, we examined the iNOS mRNA expression by northern blot analysis. Contrary to our expectation, EX-4 failed to inhibit IL-1β-induced iNOS mRNA expression (Fig. 2A). Next, we analyzed the effect of EX-4 on transactivation activities of the iNOS promoter by transient transfection experiment. Parallel to the result of iNOS mRNA expression, EX-4 did not inhibit IL-1β-induced iNOS promoter activity (Fig. 2B).

Effects of EX-4 on IL-1β-induced NF-κB activation

Since the activation of transcription factor NF-κB is an essential step for the IL-1β-induced iNOS expression, we evaluated the effect of EX-4 on binding activity of NF-κB to the iNOS promoter region (Fig. 3A). Although multiple nuclear proteins (C1–C4) formed complexes with the probe, the band intensity of C2 was strongly increased by IL-1β. By immune-supershift EMSA using anti-p65 antibody, the supershifted band was detected (arrow in Fig. 3A), which suggests that p65 NF-κB subunit binds specifically to the NF-κB binding site of the promoter. However, EX-4 did not reduce the band intensity induced by IL-1β. The majority of DNA–protein complexes were sequence-specific because a 100-fold molar excess of unlabeled wild probe completely abolished the binding activity (Fig. 3A, 1st lane). Next, we analyzed the effect of EX-4 on the nuclear translocation of p65 by IL-1β (Fig. 3B). Compared with the control group, IL-1β induced nuclear translocation of p65. However, EX-4 did not inhibit nuclear translocation of p65 by IL-1β.

Effect of EX-4 on iNOS mRNA stability

To test the role of EX-4 in the posttranscriptional regulation of iNOS involving mRNA stability, we analyzed the degradation rate of iNOS mRNA by actinomycin D chase study. EX-4 did not alter the decay rate of iNOS mRNA induced by IL-1β (Fig. 4A). To further examine the effect of EX-4 on iNOS mRNA stability, we analyzed the luciferase activity using piNOS-UTR construct containing 3′-UTR of iNOS mRNA. Under basal conditions (without any treatment), the presence of 3′-UTR reduced luciferase activity by about 0·5-fold compared with the absence of 3′-UTR (Fig. 4B1). As shown in Fig. 4B2, IL-1β significantly increased the luciferase activity of piNOS-UTR, however, EX-4 did not alter the luciferase activity induced by IL-1β.

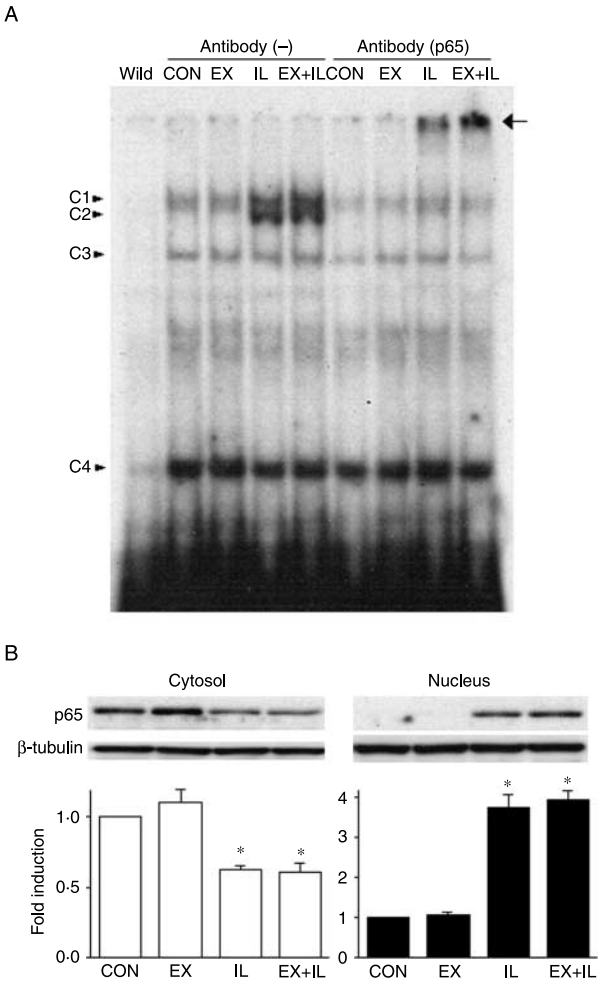

Figure 3 The effect of exendin-4 on IL-1β-induced NF-κB activation in RINm5F cells. (A) Gel mobility shift assay of NF-κB binding site on rat iNOS promoter. The cells were pretreated with exendin-4 (EX, 10 nM) for 30 min, then incubated for 30 min with IL-1β (IL, 100 pg/ml). Nuclear extracts were analyzed on mobility shift assay with NF-κB probes (5′-AGTTGAGGGGACTTTCCCAGGC-3′). C1–C4 indicates the position of major DNA-protein complexes. For supershift assay, an antibody (0·2 μg) against NF-κB subunit (p65) was incubated with nuclear extracts for 30 min before the addition of probes. The arrow indicates bands supershifted by anti-p65 NF-κB subunit. Wild, 100-fold molar excess of unlabeled NF-κB probe; CON, control without any treatment. The gel is the representative of three separate experiments with similar results. (B) The cytosolic and nuclear proteins (30 μg) were separated on a 8% SDS-PAGE and the protein levels of NF-κB subunit (p65) were measured by western blot analysis. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean±S.D. ($n=3$) and each value is expressed relative to the control value. *$P<0·05$ versus CON.

The role of cAMP in EX-4 inhibition of IL-1β-induced iNOS protein

The involvement of cAMP in iNOS protein expression was evaluated since GLP-1 agonists were reported to activate cAMP/PKA pathway via GLP-1 receptor (Drucker *et al.* 1987, Leech *et al.* 1999). Forskolin, a cAMP-increasing agent, significantly inhibited IL-1β-induced iNOS protein expression, and the inhibition of iNOS protein by EX or forskolin was reversed by PKA specific inhibitor H-89 (Fig. 5A). Next, we examined the effect of forskolin on the rat iNOS promoter activity. Likewise, EX-4 and forskolin failed to inhibit IL-1β-induced iNOS promoter activity (Fig. 5B). Based on the previous results that EX-4 induces ERK phosphorylation (Kim *et al.* 2006), the effect of MAPKs inhibitors on iNOS protein was observed. As shown in Fig. 5C, both PD98059, an MEK inhibitor and SB203580, a p38 MAPK inhibitor did not affect IL-1β-induced iNOS protein expression. To evaluate further, the role of cAMP/PKA pathway, the intracellular cAMP content and iNOS protein stability were determined. EX-4 increased cAMP production dose-dependently (0·1–10 nM), however, a higher concentration of EX-4 (100 nM) did not increase cAMP production (Fig. 5D1). Additionally, EX-4 treatment restored the IL-1β-induced decrease in cAMP level toward normal control level (Fig. 5D2). Next, to evaluate the effect of cAMP on iNOS protein stability, we performed pulse-chase experiment using cycloheximide (Fig. 5E). EX-4 significantly increased iNOS protein degradation compared with cycloheximide alone (control). Under the influence of EX-4, the half-life of iNOS protein level induced by IL-1β was about 1 h. However, in the absence of EX-4, the calculation of iNOS protein half-life was impossible because iNOS protein was only decreased by 10% even after 6 h. This finding suggests that EX-4-induced increase in cAMP level is involved in iNOS protein degradation.

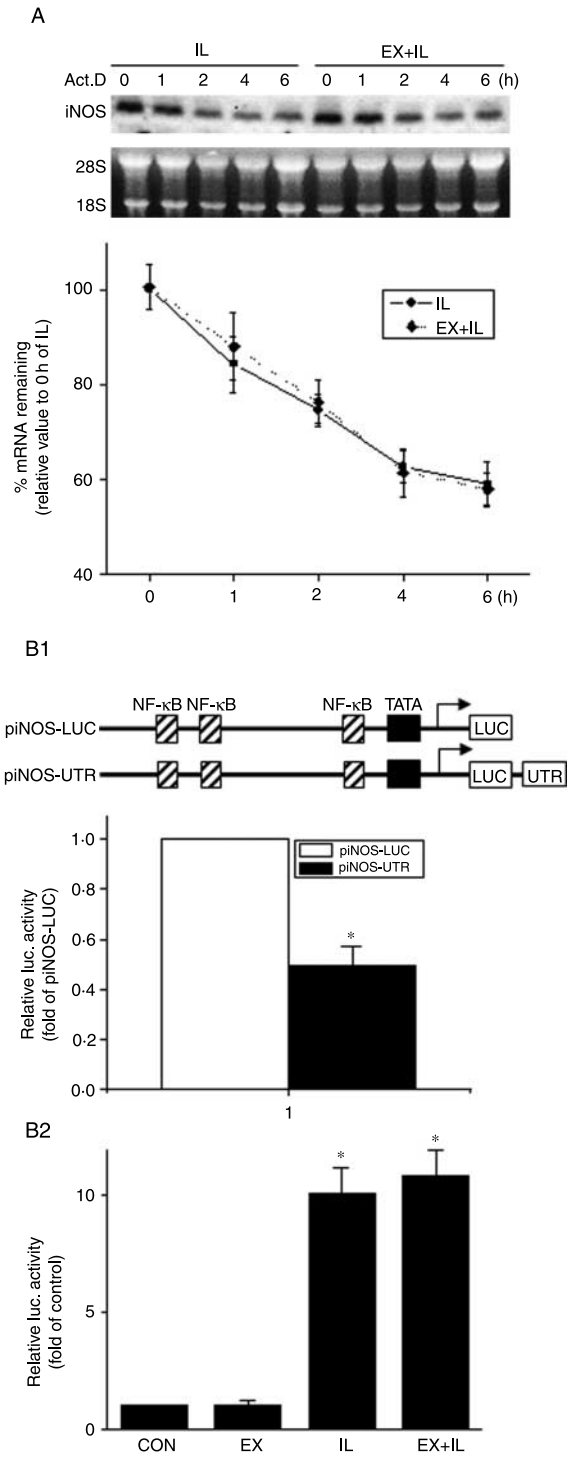

Figure 4 Effect of exendin-4 on the stability of iNOS mRNA in RINm5F cells. (A) The cells were pretreated with exendin-4 (EX, 10 nM) for 1 h, then with IL-1β (IL, 100 pg/ml). After 6 h of incubation, actinomycin D (0·5 μg/ml) was added to the cells. The expressions of iNOS mRNA were measured by Northern blot analysis. Equal loading of sample (10 μg) was verified by the ribosomal 18S and 28S bands. Data were plotted on percentage of remaining mRNA relative to 0 h and represent the mean±S.D. (n=3). Act. D, actinomycin D. (B1) Schematic representation of piNOS-UTR. The cells were transiently cotransfected with piNOS-LUC (1 μg) and pSV-β-gal (0·1 μg, as an internal control). Following the serum starvation, the cells were then solubilized in lysis buffer, and luciferase and β-galactosidase activities were measured. Data are expressed as mean±S.D. (n=4) and the value is expressed relative to the piNOS-LUC value. *P<0·05 versus piNOS-LUC. LUC, luciferase; UTR, 3′-untranslated region of rat iNOS mRNA. (B2) The effect of exendin-4 on the luciferase activity of piNOS-UTR. The cells were transiently cotransfected with piNOS-LUC (1 μg) and pSV-β-gal (0·1 μg, as an internal control). Following the serum starvation, the cells were pretreated with EX (10 nM) for 30 min, then cultured for an additional 10 h with IL (100 pg/ml). The cells were then solubilized in lysis buffer, and luciferase and β-galactosidase activities were measured. Data are expressed as mean±S.D. (n=4) and each value is expressed relative to the control value. *P<0·05 versus CON. CON, control without any treatment.

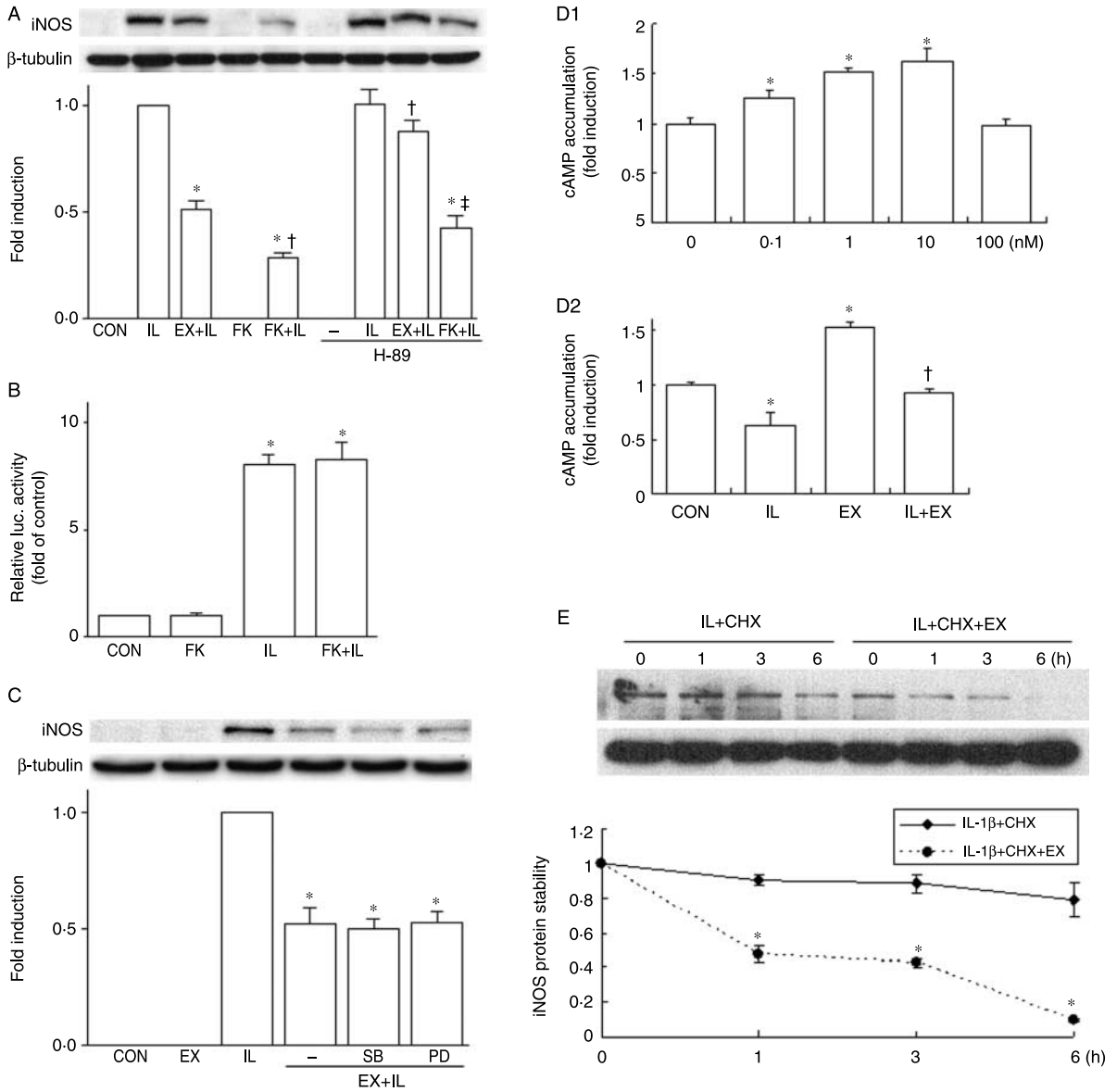

Figure 5 The role of cAMP in exendin-4 inhibition of IL-1β-induced iNOS protein in RINm5F cells. (A) The cells were pretreated with H-89 (5 μM) for 30 min, next incubated with exendin-4 (EX, 10 nM) or forskolin (FK, 10 μM) for 1 h, and then cultured with IL-1β (IL, 100 pg/ml) for an additional 16 h. Western blot analysis for iNOS was performed. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean (±S.D.) of relative band density ($n=3$). *$P<0{\cdot}05$ versus IL; †$P<0{\cdot}05$ versus EX+IL; ‡$P<0{\cdot}05$ versus FK+IL. CON, control without any treatment. (B) The effect of forskolin (FK) on IL-induced iNOS promoter activity. The cells were transiently cotransfected with piNOS-LUC (1 μg) and pSV-β-gal (0·1 μg, as an internal control). Following the serum starvation, the cells were pretreated with FK (10 μM) for 30 min, then cultured for an additional 10 h with IL (100 pg/ml). Data are expressed as mean±S.D. ($n=4$) and each value is expressed relative to the control value. *$P<0{\cdot}05$ versus CON. (C) The cells were pretreated with PD98059 (PD, 10 μM) or SB203580 (SB, 10 μM) for 30 min, next incubated with exendin-4 (EX, 10 nM) for 1 h, and then cultured with IL-1β (IL, 100 pg/ml) for an additional 16 h. Western blot analysis for iNOS was performed. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean (±S.D.) of relative band density ($n=3$). *$P<0{\cdot}05$ versus IL. (D) Measurement of cAMP content. The cells were incubated with EX-4 (10 nM) for 30 min, and then incubated with IL-1β (100 pg/ml) for 30 min. The phosphodiesterase inhibitor 3-iosbutyl-1-methylxanthine (IBMX, 250 μM) were added to prevent cAMP breakdown. The cellular cAMP content was measured using cAMP assay kit according to the manufacturer's instructions. Data are expressed as mean±S.D. ($n=4$) and each value is expressed relative to the control value. D1: *$P<0{\cdot}05$ versus without EX treatment. D2: *$P<0{\cdot}05$ versus CON; †$P<0{\cdot}05$ versus IL or EX. (E) iNOS protein stability assay. The cells were incubated with IL-1β (IL, 100 pg/ml) for 16 h, and then exposed to cycloheximide (CHX; 2 μg/ml) for 1 h to stop protein synthesis, and next treated with EX-4 (10 nM) for indicated time points. Western blot analysis for iNOS was performed. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean (±S.D.) of relative band density ($n=3$). *$P<0{\cdot}05$ versus IL only.

Discussion

The excessive production of NO via IL-1β-stimulated iNOS induction has been implicated in β-cell damage of type 1 diabetes mellitus (Corbett & McDaniel 1992, Thomas *et al.* 2002). NO is highly reactive, and readily converts to toxic peroxynitrite (Bredt & Snyder 1994). The clinical relevance of NO in the incidence of type 1 diabetes has been demonstrated in the epidemiologic studies. High intakes of nitrite and *N*-nitroso compounds were closely related with high incidence of type 1 diabetes (Kostraba *et al.* 1992, Virtanen *et al.* 1994).

It is well known that pancreatic β-cells show remarkably low antioxidant enzyme activities compared with liver or kidney (Lenzen *et al.* 1996, Tiedge *et al.* 1997). The RINm5F cell is comparable with the primary β-cells in the aspects of IL-1β-mediated NO production, iNOS expression, and cytotoxicity (Meßmer & Brüne 1994, Kwon *et al.* 1995, Larsen *et al.* 1998). We previously observed that the responses of RINm5F cells to IL-1β were similar to those of the primary isolated islets in nitrite formation and iNOS protein expression (Kim *et al.* 2003, 2004). These results strongly suggest that RINm5F cells may be an appropriate substitute for the primary β-cells. Therefore, we sought to define the actions of EX-4, a potent GLP-1 analog, on IL-1β-mediated iNOS expression mechanism employing RINm5F β-cells.

We observed that EX-4 significantly inhibited IL-1β-mediated nitrite production and iNOS protein expression consistent with previous studies, in which GLP-1 pretreatment inhibited cytokines- or high glucose-mediated iNOS protein expression in INS-1E cells or diabetic rat islets respectively (Li *et al.* 2005, Salehi *et al.* 2008). These results suggest that EX-4 may inhibit the action of IL-1β on iNOS expression at the level of protein or above.

Next, we observed the action of EX-4 on iNOS mRNA expression to examine whether the EX-4 inhibition of iNOS protein level could be attributable to the inhibition of iNOS mRNA content. Unexpectedly, EX-4 did not inhibit iNOS mRNA level induced by IL-1β. A similar finding was also reported in another study employing J774 macrophages (Irace *et al.* 2007). Oxalomalate, an inhibitor of mitochondrial aconitase, suppressed lipopolysaccharide-induced nitrite production and iNOS protein expression in J774 cells without affecting iNOS mRNA content. To confirm our result of iNOS mRNA, we analyzed the effect of EX-4 on the iNOS promoter activity since the iNOS gene induction is traditionally regulated by the transactivation of iNOS promoter through primary transcription factors including NF-κB (Kleinert *et al.* 2004). Parallel to the result of iNOS mRNA, EX-4 did not suppress the iNOS promoter activity induced by IL-1β. Like EX-4, forskolin, a cAMP-increasing agent, did not inhibit IL-1β-stimulated iNOS promoter activity (Fig. 5B) and iNOS mRNA expression (data not shown). These findings suggest that the increase in cAMP level did not inhibit IL-1β-induced iNOS at the transcriptional level. We further evaluated the effect of EX-4 on the action of NF-κB since the importance of NF-κB binding sites has been well known for the induction of iNOS promoter activity (Spink *et al.* 1995, Eberhardt *et al.* 1998). Consistent with the result of iNOS promoter study, EX-4 did not suppress IL-1β-stimulated binding activity of NF-κB to the iNOS promoter and did not inhibit IL-1β-mediated nuclear translocation of p65, an NF-κB subunit. These findings indicate that EX-4 seems to inhibit iNOS expression at the protein level, but not at the transcriptional level.

Besides the transcriptional regulation, the posttranscriptional mechanism is also involved in the induction of iNOS gene expression (Kleinert *et al.* 2004). Therefore, to further explore whether EX-4 inhibition of IL-1β-induced iNOS protein expression was attributable to the instability of iNOS mRNA following the transcription of iNOS gene, we analyzed the role of EX-4 in the posttranscriptional regulation of iNOS mRNA. Our actinomycin D chase study shows that EX-4 did not affect the degradation rate of iNOS mRNA induced by IL-1β. The 3′-UTR of rat iNOS mRNA has six AU-rich elements (ARE, AUUUA), which interact with ARE-binding proteins including HuR and polypyrimidine tract-binding protein, contributing to iNOS mRNA stability (Kleinert *et al.* 2004, Pautz *et al.* 2006). Therefore, we analyzed the effect of EX-4 on iNOS mRNA stability using 3′-UTR-containing luciferase construct (Fig. 4B). In this study, the presence of 3′-UTR significantly reduced the luciferase activity in basal conditions while the treatment of IL-1β profoundly increased the luciferase activity. These findings are supported by other studies. In the human iNOS, 3′-UTR affected the gene regulation by decreasing its basal expression, however, 3′-UTR significantly induced the gene expression upon the treatment of cytokine mixture (Nunokawa *et al.* 1997). Consistent with the result of actinomycin D chase experiment, EX-4 did not affect the luciferase activity induced by IL-1β. Therefore, EX-4 may not affect iNOS gene expression at the posttranscriptional level.

The involvement of proteasome in iNOS protein degradation has been suggested in some cells. In mouse peritoneal macrophage, transforming growth factor-β reduced IFN-γ-stimulated iNOS protein level via increased degradation of the protein (Vodovotz *et al.* 1993). The iNOS protein is primarily regulated by proteasome pathway in Raw264·7 and HEK294 cells (Walker *et al.* 1997, Musial & Eissa 2001, Kolodziejski *et al.* 2002). However, in islet β-cells, knowledge concerning the role of ubiquitin-proteasome pathway in iNOS protein degradation is very limited. Pituitary adenylate cyclase-activating polypeptide 27, a cAMP-producing peptide suppressed iNOS expression in lipid-infused rat islets, which was not reversed by the treatment of a proteasome inhibitor MG 132 (Qader *et al.* 2007). Additionally, MG132 did not reverse the GLP-1-induced suppression of iNOS expression in diabetic GK rat islets, but instead MG132 induced loss of iNOS protein (Salehi *et al.* 2008). These findings suggest that the

suppressive effect of cAMP-increasing agents on iNOS protein expression may be exerted via non-proteasomal mechanisms. Moreover, GLP-1 inhibited IFN-γ-induced NO production via suppression of TNF-α production in MIN6N8a cells (Hahm *et al.* 2008). Therefore, to clarify the mechanism of EX-4 inhibition of IL-1β-induced iNOS protein expression, further studies including both proteasomal and non-proteasomal pathways will be required.

Growing evidence suggests that cAMP inhibits most inflammatory reactions such as the production of inflammatory cytokines and oxidative burst (Ottonello *et al.* 1995, Willis & Nisen 1995). However, the role of cAMP in iNOS expression is different depending on the cell types (Galea & Feinstein 1999). In rat, peritoneal macrophages and macrophage cell lines, cAMP upregulated iNOS expression (Sowa & Przewlocki 1994, Jeon *et al.* 1996) or inhibited it (Marotta *et al.* 1992, Pang & Hoult 1997). Moreover, cAMP potentiated iNOS expression in aortic smooth muscle (Koide *et al.* 1993), brown adipose tissue (Nisoli *et al.* 1997), and brain endothelium (Durieu-Trautmann *et al.* 1993) while cAMP decreased iNOS expression in pancreatic cells (Andersen *et al.* 1996) and rat hepatocytes (Smith *et al.* 1997).

Therefore, we examined the involvement of signaling molecules in the EX-4 inhibition of IL-1β-induced iNOS protein since cAMP/PKA system induced by GLP-1 or EX-4 is a critical signaling pathway for β-cell survival and growth (Jhala *et al.* 2003). The present study showed that forskolin, a cAMP increasing agent significantly inhibited IL-1β-induced iNOS protein expression and that the inhibitory effects of EX-4 and forskolin were reversed by H-89, a PKA inhibitor (Fig. 5A). Dibutyryl-cAMP, a membrane permeable cAMP analog also had a similar effect to forskolin (data not shown). These findings are consistent with others' reports. GLP-1 suppressed high glucose-stimulated iNOS expression in normal islet tissue and excessive expression of iNOS in diabetic GK rat islets, and these effects were reversed by PKA inhibition (Jimenez-Feltstrom *et al.* 2005, Salehi *et al.* 2008). A few previous studies, though not the study of GLP-1, indicate that cAMP/PKA signaling pathway was involved in IL-1β-induced iNOS expression. IL-1-induced nitrite production was antagonized by lipophilic cAMP such as CPT-cAMP and 8-Br-cAMP (Meßmer & Brüne 1994). In the isolated islets, IL-1β-induced nitrite production was reduced by 3-isobutyl-1-methyl xanthine, a phosphodiesterase inhibitor (Andersen *et al.* 1996).

Meanwhile, GLP-1 and EX-4 have been known to induce the phosphorylation of ERK in β-cells (Buteau *et al.* 2001, Arnette *et al.* 2003, Kim *et al.* 2006), and so we tested ERK pathway in iNOS protein expression. PD98059, an MEK inhibitor did not alter IL-1β-induced iNOS protein expression, which is supported by another study that found that the inhibition of MAP kinase pathway by PD98059 had no effect on the anti-apoptotic function of liraglutide, a long-acting GLP-1 analog (Bregenholt *et al.* 2005).

To further evaluate the role of cAMP/PKA pathway in iNOS protein expression, intracellular cAMP content was measured. EX-4 produced cAMP formation dose-dependently (0·1–10 nM), however, a higher concentration of EX-4 (100 nM) failed to increase cAMP production (Fig. 5D1). These findings were consistent with the results of another study (Sidhu *et al.* 2005). Additionally, EX-4 treatment restored the IL-1β-induced decrease in cAMP level toward normal control level (Fig. 5D2). Based on this finding, we observed iNOS protein stability using cycloheximide to identify the role of cAMP in iNOS protein degradation (Fig. 5E). EX-4 significantly increased iNOS protein degradation compared with control. Forskolin also showed a similar effect to EX-4 (data not shown). Accordingly, the present findings indicate that the increase in cAMP level seems to be involved in iNOS protein degradation by EX-4.

In conclusion, we suppose that the EX-4-activated cAMP/PKA pathway may be involved in the suppression of IL-1β-induced iNOS expression at posttranslational level (iNOS protein stability) without affecting transcriptional and posttranscriptional levels.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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