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Exendin-4 Inhibits iNOS Expression at the Protein Level in LPS-Stimulated Raw264.7 Macrophage by the Activation of cAMP/PKA Pathway

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) and its potent agonists have been widely studied in pancreatic islet β-cells. However, GLP-1 receptors are present in many extrapancreatic tissues including macrophages, and thus GLP-1 may have diverse actions in these tissues and cells. Therefore, we examined the mechanism by which exendin-4 (EX-4), a potent GLP-1 receptor agonist, inhibits lipopolysaccharide (LPS)-induced iNOS expression in Raw264.7 macrophage cells. EX-4 significantly inhibited LPS-induced iNOS protein expression and nitrite production. However, Northern blot and promoter analyses demonstrated that EX-4 did not inhibit LPS-induced iNOS mRNA expression and iNOS promoter activity. Electrophoretic mobility shift assay (EMSA) showed that EX-4 did not alter the binding activity of NF-κB to the iNOS promoter. Consistent with the result of EMSA, LPS-induced IκBα phosphorylation and nuclear translocation of p65 were not inhibited by EX-4. Also, actinomycin D chase study and the promoter assay using the construct containing 3′-untranslated region of iNOS showed that EX-4 did not affect iNOS mRNA stability. Meanwhile, cycloheximide chase study demonstrated that EX-4 significantly accelerated iNOS protein degradation. The EX-4 inhibition of LPS-induced iNOS protein was significantly reversed by adenylate cyclase inhibitors (MDL-12330A and SQ 22536), a PKA inhibitor (H-89) and PKAα gene silencing. These findings suggest that EX-4 inhibited LPS-induced iNOS expression at protein level, but not at transcriptional mechanism of iNOS gene and this inhibitory effect of EX-4 was mainly dependent on cAMP/PKA system. J. Cell. Biochem. 114: 844–853, 2013.

KEY WORDS: EXENDIN-4 (EX-4); LPS; iNOS; NF-κB; cAMP/PKA; RAW264.7 MACROPHAGE

Activated macrophages play a pivotal role in inflammatory responses by producing inflammatory markers such as cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 [Laskin and Pendino, 1995]. The excessive production of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ lead to the overproduction of toxic molecules including nitric oxide (NO). NO is excessively synthesized by iNOS in macrophages exposed to LPS and inflammatory cytokines, and reactive nitrogen species are implicated in various diseases such as autoimmune diabetes, septic shock and rheumatoid arthritis [O'Shea et al., 2002].

Glucagon-like peptide-1 (GLP-1) and its potent agonist exendin-4 (EX-4) have received great attention because of their insulinotropic and proliferative effects in β-cells [Bulotta et al., 2004]. Both

Abbreviations used: EX-4, exendin-4; GLP-1, glucagon-like peptide-1; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NF-κB, nuclear factor-κB; PKA, protein kinase A.

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

Seo-Yoon Chang and Dong-Bin Kim contributed equally to this work.

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GLP-1 and EX-4 activate multiple signaling pathways such as cAMP/PKA, phosphatidylinositol 3-kinase and mitogen-activated protein kinase, which lead to the islet mass increase and β-cell growth [Jhala et al., 2003; List and Habener, 2004]. For these effects, synthetic version of EX-4, exenatide (marketed as Byetta, Amylin Pharmaceuticals and Eli Lilly) has been applied to the treatment of type 2 diabetes mellitus.

The extrapancreatic actions of GLP-1 and EX-4 have been reported elsewhere. The cardioprotective and cardiotropic effects of GLP-1 have been shown in animal and human studies [Yamamoto et al., 2002; Sokos et al., 2006]. GLP-1 and EX-4 increase hepatic glucose uptake and glycogen synthesis, and may mediate the regulation of hepatic glucose output by insulin [D'Alessio et al., 2004; Zheng et al., 2009]. GLP-1 is also synthesized in the solitary tract nucleus, and GLP-1 receptor is widely distributed in the brain. Therefore, GLP-1 is known to be involved in the regulation of appetite as well as neuroprotective action [Drucker, 2006].

Recently, GLP-1 receptor was known to be located in monocyte/macrophage cells and EX-4 inhibited the expressions of inflammatory mediators TNF-α and monocyte chemoattractant protein-1 in LPS-activated mouse peritoneal macrophages [Arakawa et al., 2010]. Additionally, EX-4 reduced the release of TNF-α and IL-1β from high glucose-stimulated THP-1 macrophage cells [Kodera et al., 2011]. However, little is known about the action of EX-4 on iNOS expression in macrophage cells. Therefore, we investigated molecular mechanisms by which EX-4 regulates iNOS expression in LPS-activated Raw264.7 macrophage cells.

MATERIALS AND METHODS

MATERIALS

Mouse Raw264.7 cells were obtained from American Type Tissue Collection (Rockville, MD). Exendin-4 (EX-4) was from Bachem AG (Torrance, CA). Forskolin, H-89 and cycloheximide were from Calbiochem (La Jolla, CA). Lipofectamine^{TM} 2000 reagent was from Invitrogen (Carlsbad, CA). Bio-Rad protein assay kit was from BIO-RAD (Richmond, CA). Griess Reagent System, pGL3-Basic Vector, and pRL-TK were from Promega (Madison, WI). Anti-mouse iNOS antibody was from BD Transduction Laboratories (Palo Alto, CA). Anti-rabbit IκBα antibody and anti-mouse phospho-IκBα antibody were from Cell Signaling Technologies (Danvers, MA). PKAα siRNA (sc-36241) and anti-p65 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma (St. Louis, MO). EX-4 was prepared in distilled water. Forskolin and H-89 were dissolved in DMSO at 10 mM stock solution. The final concentration of vehicles did not exceed 0.1%.

CELL CULTURE

Raw264.7 cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), and the cells were maintained at 37°C in a carbon dioxide incubator under a humidified atmosphere of 5% CO_2 and 95% air. Cells were subcultured weekly and passages 4–13 were used exclusively. We fixed the concentration (100 ng/ml) of LPS based on our preliminary experiment. The cells were plated at a concentration of 3×10^5/ml except for transient transfection experiment. At 80% confluency, the cells were employed in various experiments.

MEASUREMENT OF NO AS NITRITE

Raw264.7 cells were pretreated with EX-4 (10 nM) for 1 h, next incubated with LPS (100 ng/ml) for an additional 16 h. Released nitrite was measured using Griess Reagent System as described previously [Kang et al., 2009]. 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). Nitrite concentrations were determined from a calibration curve of standard $NaNO_2$ concentrations against absorbance. Data are adjusted by protein concentration to correct differences among the groups and each value is expressed relative to the control value.

WESTERN BLOT ANALYSIS

Western blot analysis was performed essentially as described previously [Kim et al., 2004, 2006]. The cells were harvested, and solubilized with RIPA buffer. The soluble fraction was collected, and protein content was determined by Bio-Rad protein assay kit. Thirty microgram of total protein was separated on an 8% SDS–PAGE and transferred onto nitrocellulose membrane. After blocking with skimmed milk, the membrane was incubated with anti-mouse iNOS IgG (0.5 μg/ml in TTBS). Then, the membrane was probed with peroxidase-conjugated anti-mouse IgG (0.5 μg/ml in TTBS). The signal was visualized by enhanced chemiluminescence system. The immunoreactive band intensity was determined by densitometry using Scion Image program (Scion Corporation, Frederick, MD). Each value was normalized by the ratio of iNOS band intensity to β-tubulin band intensity. Data are expressed as mean ± SD of four independent experiments and each data is expressed relative to control value. For the measurement of IκBα phosphorylation, Western blot analysis was performed using IκBα and phospho-IκBα antibodies by the same procedure as described above. In addition, for the nuclear translocation of p65, nuclear and cytosolic fractions were obtained using sequential hypotonic and hypertonic solutions. Western blot analysis was performed using p65 antibody by the same procedure as described above. Equal loading and transfer of samples were verified by the band intensity of β-tubulin or TATA-binding protein.

NORTHERN BLOT ANALYSIS

Raw264.7 cells were treated with EX-4 (10 nM) for 1 h, and then incubated with LPS (100 ng/ml) for an additional 6 h. Total RNA isolation and the method for Northern blot analysis were described previously [Kang et al., 2006, 2008]. The preparation of iNOS probe was demonstrated in the previous study [Kim et al., 2004]. Equal loading of sample was verified by the ribosomal 18S band.

PLASMIDS

Mouse genomic DNA was prepared from Raw264.7 cells using AccuPrep genomic DNA extraction kit according to the manufacturer's instruction. The mouse iNOS promoter region between

nucleotides −1102 and +111 was amplified by PCR on 250 ng genomic DNA with GoTaq® DNA Polymerase. Primers were as follows (GenBank Accession No: L23806.1): forward, from −1102, 5′-TCAGGTACCTCTCCCAACTATTGAGGCC-3′ with *Kpn*I site (underlined); reverse, from +111, 5′-TCACTCGAGAAGTGTCTC-TAACAGCTCAGTC-3′ with *Xho*I site (underlined). The PCR product was purified from agarose gel, digested, and cloned into *KpnI* and *Xho*I sites of pGL3-Basic vector to obtain piNOS-Luc plasmid construct. The construct was verified by sequencing using An ABI PRISM 310 genetic analyzer (PerkinElmer Ltd. Co, Seoul, Korea). The pRiNOS-LUC-3′UTR, which detects iNOS mRNA stabilization, was kindly provided by Dr. M. Nishizawa (Department of Biomedical Sciences, Ritsumeikan University, Japan). The construction of this plasmid was well described in Dr. Nishizawa's previous studies [Matsui et al., 2008; Ozaki et al., 2010].

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAY

Transient transfection was performed by lipofection using Lipofectamine™ 2000 reagent as described previously [Kim et al., 2006]. Briefly, Raw264.7 cells were plated at a density of 2×10^5/well in a 12-well plate 1 day before the transfection. Cells at 70% confluence were cotransfected with piNOS-LUC (0.5 μg) and pRL-TK (50 ng). After 8 h, the cells were treated with EX-4 (10 nM) for 1 h, and then incubated with LPS (100 ng/ml) for an additional 16 h, and then harvested for determination of the firefly and Renilla luciferase activities. All data were normalized by the ratio of firefly to Renilla luciferase activities.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Raw264.7 cells were treated with EX-4 (10 nM) for 30 min, and then incubated with LPS (100 ng/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 mouse iNOS promoter by the method reported previously [Kim et al., 2004, 2006]. For the DNA binding activity of NF-κB, the oligonucleotide probe (5′-CCAACTGGGGACTCTCCCTTTGGGA-3′) containing a κB binding sites (underlined) was used. The antibodies against NF-κB subunits p65 and p50 were used for immune-supershift assay.

INOS MRNA STABILITY ASSAY

Raw264.7 cells were treated with EX-4 (10 nM) for 1 h, and then incubated with LPS (100 ng/ml) for 6 h, and next exposed to actinomycin D (0.5 μ/ml) to block RNA synthesis. The cells were harvested at time points of 0, 1, 2, and 4 h after the addition of actinomycin D. Total RNA was extracted and Northern blot analysis for iNOS mRNA was performed.

iNOS PROTEIN STABILITY STUDY

Raw264.7 cells were incubated with LPS (100 ng/ml) for 16 h, and then exposed to cycloheximide (1 μg/ml) for 1 h to stop protein synthesis, and next treated with EX-4 (10 nM) or forskolin (10 μM). The cells were harvested at time points of 0, 1, 2, and 4 h after the addition of EX-4 or forskolin. Total protein was extracted and Western blot analysis for iNOS protein was performed.

siRNA-MEDIATED KNOCKDOWN STUDY

PKA expression was silenced in Raw264.7 cells using target-specific 20–25 nucleotide PKAα siRNA duplexes as described previously [Kim et al., 2008]. The day before transfection, the cells were seeded in 6-well plates and grown overnight to reach 50–60% confluence. The day of the experiment, siRNA duplexes were prepared and transfection was performed according to the manufacturer's instructions. Concentration of PKAα siRNA duplexes for transfection required optimization. The final concentration of siRNA was 50 nM in each well. The cells were transfected with PKAα or control scramble siRNA (which corresponds to a nontargeting 20–25 nucleotide siRNA as a negative control), then 24 h after transfection, cells were stimulated with LPS (100 ng/ml) for 16 h and next treated with EX-4 (10 nM) for 6 h and iNOS protein expression was observed by Western blot analysis.

STATISTICAL ANALYSIS

The relative band densities were quantified using Scion Imaging software (Scion Corporation). All data obtained from each experiment were expressed as mean ± SD. The data were analyzed using one-way ANOVA with Origin 7.0 software (Microcal Software, Northampton, MA). 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 EXENDIN-4 ON LPS-INDUCED iNOS PROTEIN AND NITRITE PRODUCTION

We examined the expression level of GLP-1 receptor in macrophage cells. GLP-1 receptor was abundantly, though not comparable to islet beta-cells, expressed in macrophage cells Raw264.7 and THP-1 (Fig. 1A). This finding suggests that the interaction of GLP-1–GLP-1 receptor plays a role in macrophage function. We observed the effects of EX-4 on LPS-induced iNOS protein expression and nitrite production. EX-4 significantly inhibited LPS-induced iNOS protein expression at the concentrations of 1 and 10 nM, however, no further inhibition was observed at 100 nM (Fig. 1B). Accordingly, the concentration of EX-4 was set at 10 nM in subsequent experiments. Parallel to the result of iNOS expression, EX-4 significantly inhibited LPS-induced nitrite production (Fig. 1C).

EFFECTS OF EXENDIN-4 ON LPS-INDUCED iNOS mRNA AND PROMOTER ACTIVITY

To determine whether the inhibitory effect of EX-4 on LPS-induced iNOS protein could result from the inhibition of iNOS gene transcription, we examined the iNOS mRNA expression. Contrary to our expectation, EX-4 failed to inhibit LPS-induced iNOS mRNA expression (Fig. 2A). Next, we analyzed the effect of EX-4 on transactivation activities of the iNOS promoter using transfection experiment. Consistent with the result of iNOS mRNA expression, EX-4 did not inhibit LPS-induced iNOS promoter activity (Fig. 2B).

EFFECTS OF EXENDIN-4 ON LPS-INDUCED NF-κB ACTIVATION

Since the IκB degradation and subsequent activation of NF-κB is an essential step for iNOS expression, we evaluated the effect of EX-4

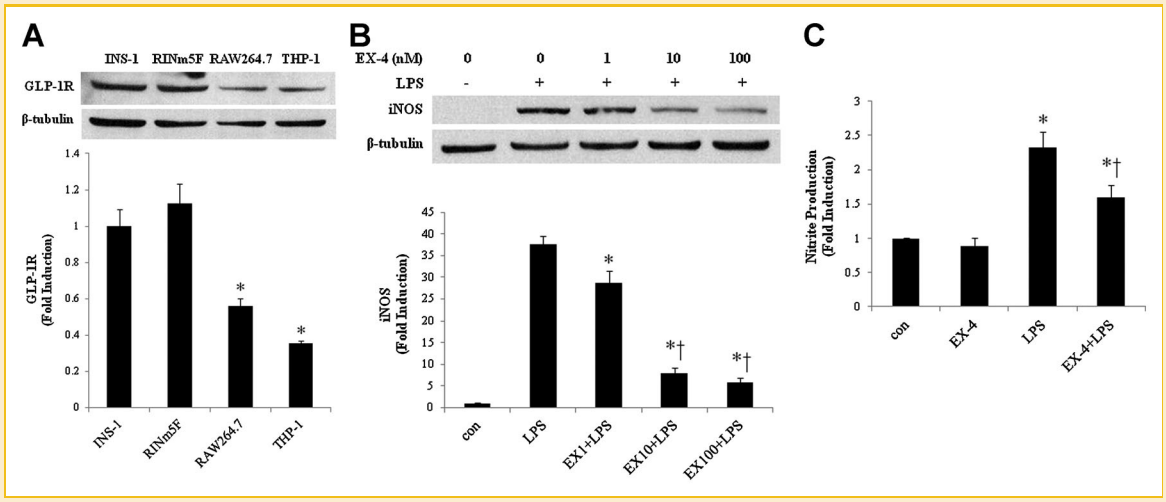

Fig. 1. The effects of exendin-4 on LPS-induced nitrite formation and iNOS protein expression in Raw264.7 cells. A: The expression level of GLP-1 receptor (GLP-1R) protein in Raw264.7 cells. Pancreatic β-cells (INS-1, RINm5F cells) and β-tubulin were employed as a positive control and a loading control, respectively. $^{*}P < 0.05$ versus INS-1. B: Raw264.7 cells were pretreated with exendin-4 (EX-4, 1, 10, and 100 nM) for 1 h, then the cells were incubated with LPS (100 ng/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 ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus LPS; $^{\dagger}P < 0.05$ versus 1 nM of EX-4 con, control without any treatment. C: Raw264.7 cells were pretreated with EX-4 (10 nM) for 1 h, and then were incubated with LPS (100 ng/ml) for an additional 16 h. Nitrite production was measured. Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus con; $^{\dagger}P < 0.05$ versus LPS. con, control without any treatment.

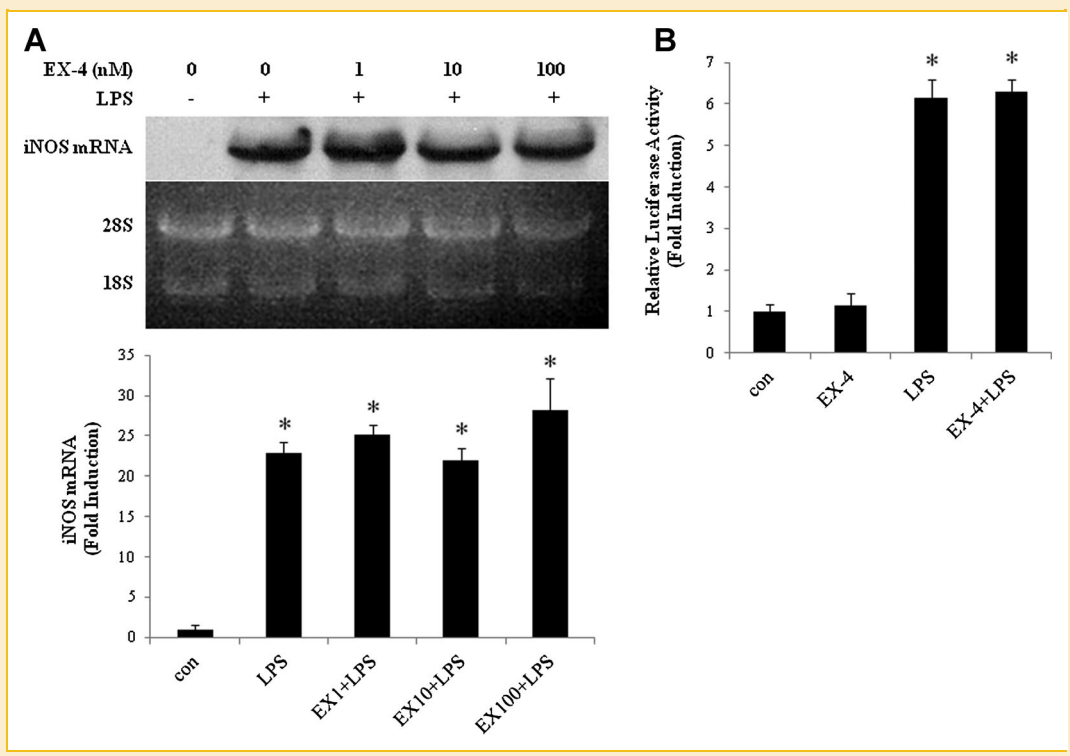

Fig. 2. The effect of exendin-4 on LPS-induced iNOS mRNA expression and iNOS promoter activity in Raw264.7 cells. A: The cells were pretreated with exendin-4 (EX-4, 1, 10, and 100 nM) for 1 h, next incubated with LPS (100 ng/ml) for an additional 6 h. The expressions of iNOS mRNA were measured by Northern blot analysis. Equal loading of sample (10 μg) was verified by the 18S ribosomal RNA band density. Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus con. con, control without any treatment. B: The cells were transiently cotransfected with piNOS-LUC (0.5 μg) and pRL-TK (50 ng, as an internal control). After 8 h, the cells were pretreated with EX-4 (10 nM) for 1 h and then incubated with LPS (100 ng/ml) for an additional 16 h. Cells were then solubilized in lysis buffer and then harvested for determination of the firefly and Renilla luciferase activities. All data were normalized by the ratio of firefly to Renilla luciferase activities. Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus con or EX-4. con, control without any treatment.

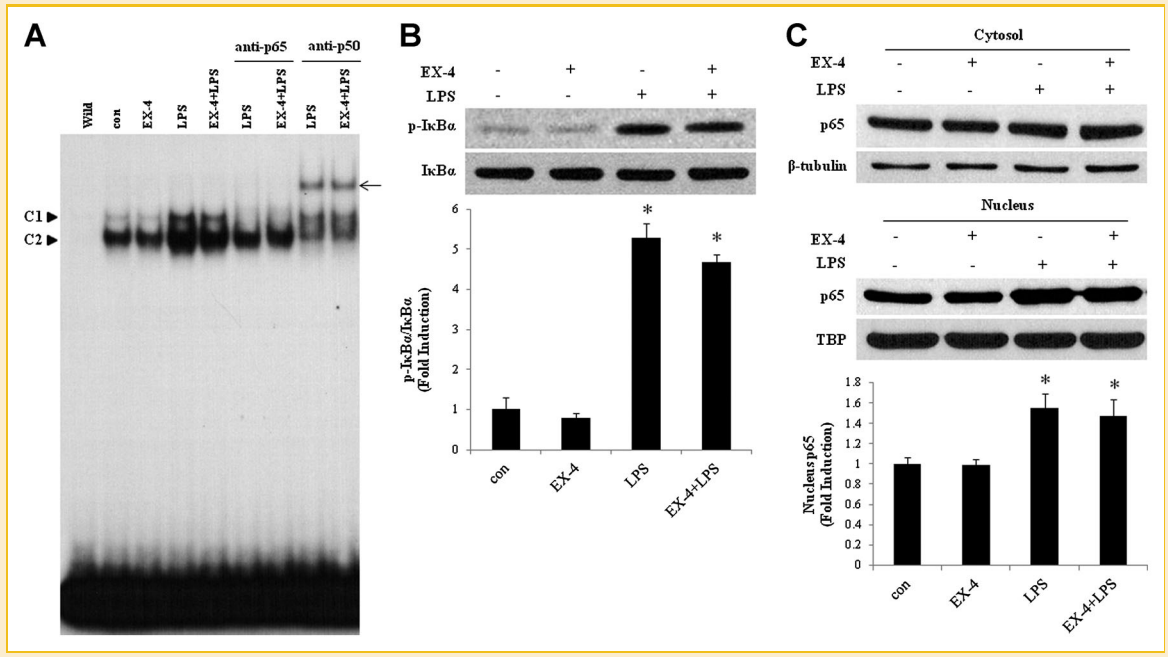

Fig. 3. The effect of exendin-4 on LPS-induced NF-κB activation in Raw264.7 cells. A: Gel mobility shift assay of NF-κB binding site on mouse iNOS promoter. The cells were pretreated with exendin-4 (EX-4, 10 nM) for 30 min, then incubated for 30 min with LPS (100 ng/ml). Nuclear extracts from were analyzed on mobility shift assay with a NF-κB probes (5′-CCAACTGGGGACTCTCCCTTTGGGA-3′). The C1 and C2 indicate the position of major DNA–protein complexes. For supershift assay, the antibody (0.2 μg) against NF-κB subunit (p65 and p50) was incubated with nuclear extracts for 30 min before the addition of probes. The arrow indicates band supershifted by anti-p50 NF-κB subunit. Wild: 100-fold molar excess of unlabeled NF-κB probe; con, control without any treatment. The gel is the representative of four separate experiments with similar results. B: The cells were pretreated with exendin-4 (EX-4, 10 nM) for 30 min, and then incubated for 30 min with LPS (100 ng/ml). Western blot analyses for IκBα and phospho-IκBα were performed. Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus con or EX-4. con, control without any treatment. C: The cells were pretreated with exendin-4 (EX-4, 10 nM) for 30 min, and then incubated for 30 min with LPS (100 ng/ml). The cytosolic and nuclear proteins (30 μg) were separated on a 10% SDS–PAGE and the protein levels of NF-κB subunit (p65) were measured. Equal loading of protein was verified by probing the same blot for β-tubulin or TATA-binding protein (TBP). Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus con or EX-4. con, control without any treatment.

on binding activity of NF-κB to the iNOS promoter (Fig. 3A). Two main DNA–protein complex bands (C1 and C2) were detected in untreated control groups, and these bands were strongly increased by LPS. However, in line with the result of promoter activity, EX-4 did not reduce LPS-induced band intensity. By immune-supershift EMSA, C1 band was abolished by preincubation with anti-p65 antibody and C2 band was supershifted by anti-p50 antibody, which suggests that NF-κB subunits bind specifically to the NF-κB binding site of the promoter. 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). As shown in Figure 3B, LPS significantly increased IκBα phosphorylation in Raw264.7 cells. However, the preincubation with EX-4 did not inhibit the LPS-induced IκBα phosphorylation. Also, EX-4 did not suppress LPS-induced nuclear translocation of p65 NF-κB subunit (Fig. 3C). These results suggest that EX-4 do not inhibit iNOS expression in LPS-treated Raw264.7 cells by a transcriptional mechanism.

EFFECT OF EXENDIN-4 ON iNOS MRNA STABILITY

Besides the transcriptional regulation, the posttranscriptional mechanism is known to be involved in iNOS gene expression [Kleinert et al., 2004]. Therefore, to test the role of exendin-4 in the posttranscriptional regulation of iNOS expression, we analyzed the degradation rate of iNOS mRNA using actinomycin D. Compared with control group, EX-4 did not change the decay rate of iNOS mRNA induced by LPS (Fig. 4A). Also, we analyzed the effect of EX-4 on iNOS mRNA stability using pRiNOS-LUC-3′UTR, which contains 3′-UTR of iNOS mRNA. As shown in Figure 4B, EX-4 did not alter the luciferase activity induced by LPS treatment. These findings indicate that EX-4 did not affect iNOS mRNA stability in LPS-treated Raw264.7 cells.

EFFECT OF EXENDIN-4 ON iNOS PROTEIN STABILITY

Since the posttranslational regulation is involved in iNOS gene expression, we evaluated the effect of EX-4 on iNOS protein expression. First, we examined the effect of EX-4 on iNOS protein stability using cycloheximide, a protein synthase inhibitor. As shown in Figure 5A, EX-4 inhibited LPS-induced iNOS protein expression (lanes 1 and 2). Cycloheximide treatment significantly inhibited LPS-induced iNOS protein expression and the addition of EX-4 further augmented the cycloheximide effect (lanes 3 and 4). EX-4 is well known to increase intracellular cAMP concentration by activation of adenylate cyclase. Therefore, to examine the involvement of cAMP in the destabilizing effect of EX-4 on iNOS protein, we employed adenylate cyclase inhibitors, MDL-12330A

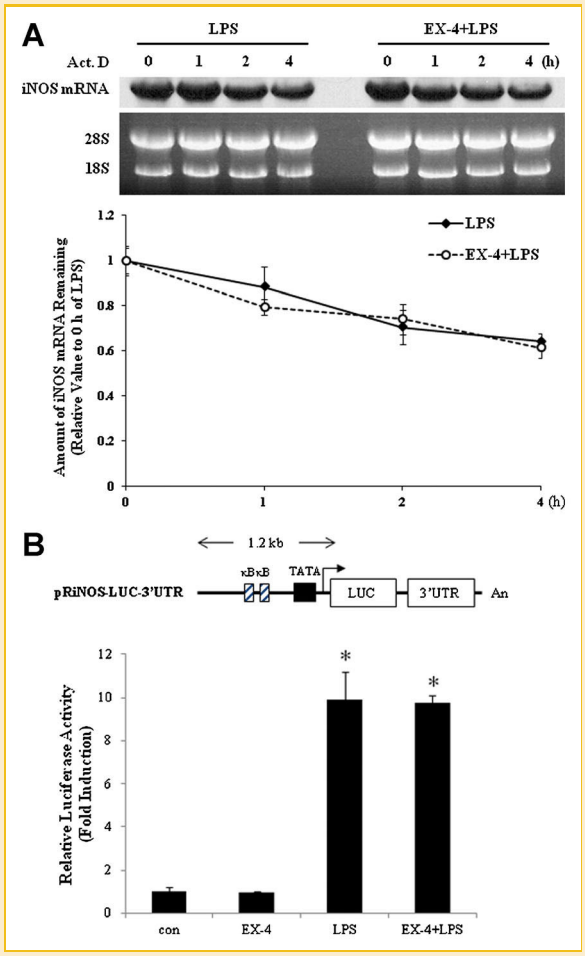

Fig. 4. Effect of exendin-4 on iNOS mRNA stability in Raw264.7 cells. A: The cells were pretreated with LPS (100 ng/ml) for 6 h and then were treated actinomycin D (Act. D, 0.5 μg/ml) for 1 h, and then incubated with exendin-4 (EX-4, 10 nM) for indicated times. The expressions of iNOS mRNA were measured by Northern blot analysis. Equal loading of sample (10 μg) was verified by the 18S ribosomal RNA band density. Data are plotted on amount of remaining mRNA relative to 0 h of LPS and represent the mean ± SD of four independent experiments. B: Schematic representation of pRiNOS-LUC-3′UTR and the effect of exendin-4 on the luciferase activity of pRiNOS-LUC-3′UTR. The cells were transiently cotransfected with pRiNOS-LUC-3′UTR (0.5 μg) and pRL-TK (50 μg, as an internal control). Following the serum starvation, the cells were pretreated with exendin-4 (EX-4, 10 nM) for 30 min, then cultured for an additional 16 h with LPS (100 ng/ml). Cells were then solubilized in lysis buffer and then harvested for determination of the firefly and Renilla luciferase activities. All data were normalized by the ratio of firefly to Renilla luciferase activities. Data are expressed as mean ± SD (n = 4) and each value is expressed relative to the control value. *$P < 0.05$ versus con. con, control without any treatment. κB, NF-κB binding site; LUC, luciferase; 3′UTR, 3′-untranslated region.

and SQ 22536 and these inhibitors restored or even further increased the iNOS protein expression inhibited by EX-4 treatment (Fig. 5B). This finding suggests that cAMP is involved in iNOS protein stability. To further analyze the effect of cAMP on iNOS protein stability, we examined time-dependent iNOS protein expression using forskolin following cycloheximide treatment. As shown in Figure 5C, forskolin significantly increased the degradation of iNOS protein compared with EX-4 after 4 h (~90% vs. ~75%). However, in the absence of EX-4 or forskolin, degradation of iNOS protein was slight after 4 h (~25%). Since the increase in intracellular cAMP by EX-4 activates protein kinase A (PKA) pathway, the involvement of PKA in iNOS protein expression was examined. H-89, a PKA inhibitor completely blocked the inhibitory effect of EX-4 on LPS-induced iNOS protein expression (Fig. 5D). In addition, PKAα gene silencing significantly prevented the inhibitory effect of EX-4 on LPS-induced iNOS protein expression (Fig. 5E). Therefore, these findings suggest that the activation of cAMP/PKA pathway by EX-4 plays a role in iNOS protein degradation.

DISCUSSION

The present study first investigated the action of EX-4, a potent GLP-1 receptor agonist on iNOS expression mechanism in LPS-stimulated Raw264.7 macrophage cells, and demonstrated that EX-4-mediated cAMP/PKA system is involved in the degradation of iNOS protein.

In Raw264.7 cells, LPS was well known to induce iNOS transcription and translation, and the overproduction of NO via the NF-κB activation pathway [Li and Verma, 2002]. The highly reactive free radical NO is a main inflammatory molecule in activated macrophage and is involved in various pathological processes including inflammation [Bogdan, 2001]. Accordingly, Raw264.7 cell used in this study is a good model for testing the effect of EX-4 on iNOS induction pathway.

EX-4 significantly inhibited LPS-stimulated nitrite production and iNOS protein expression consistent with others' studies that GLP-1 suppressed 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], and EX-4 significantly inhibited IL-1β-induced iNOS protein in INS-1 cells [Kang et al., 2009]. These results suggest that EX-4 may inhibit iNOS expression at the protein level or above.

Next, we observed the effect of EX-4 on iNOS mRNA expression. However, EX-4 did not inhibit iNOS mRNA level induced by LPS. Although this discrepancy between iNOS protein and mRNA expression pattern cannot be explained at the present time, several similar findings were reported elsewhere. Rapamycin and oxalomalate, an inhibitor of mitochondrial aconitase suppressed LPS-induced iNOS protein expression in macrophage cells without altering iNOS mRNA level [Irace et al., 2007; Jin et al., 2009]. Also, in our previous study employing beta-cells, EX-4 inhibited IL-1β-induced iNOS protein without affecting iNOS mRNA expression [Kang et al., 2009]. To further evaluate the effect of EX-4 on iNOS transcription, we examined the iNOS promoter activity since the iNOS gene expression is generally regulated by the transactivation of iNOS promoter [Kleinert et al., 2004]. Parallel to the result of iNOS mRNA, EX-4 did not suppress LPS-induced iNOS promoter activity. Next, we examined the effect of EX-4 on nuclear translocation of NF-κB, a main transcription factor for the induction of iNOS gene transcription [Spink et al., 1995]. In this study, EX-4 did not inhibit LPS-mediated IκBα phosphorylation and nuclear translocation of p65, an NF-κB subunit. Similar finding was also reported in others' studies that PPARα agonists and rapamycin inhibited iNOS protein

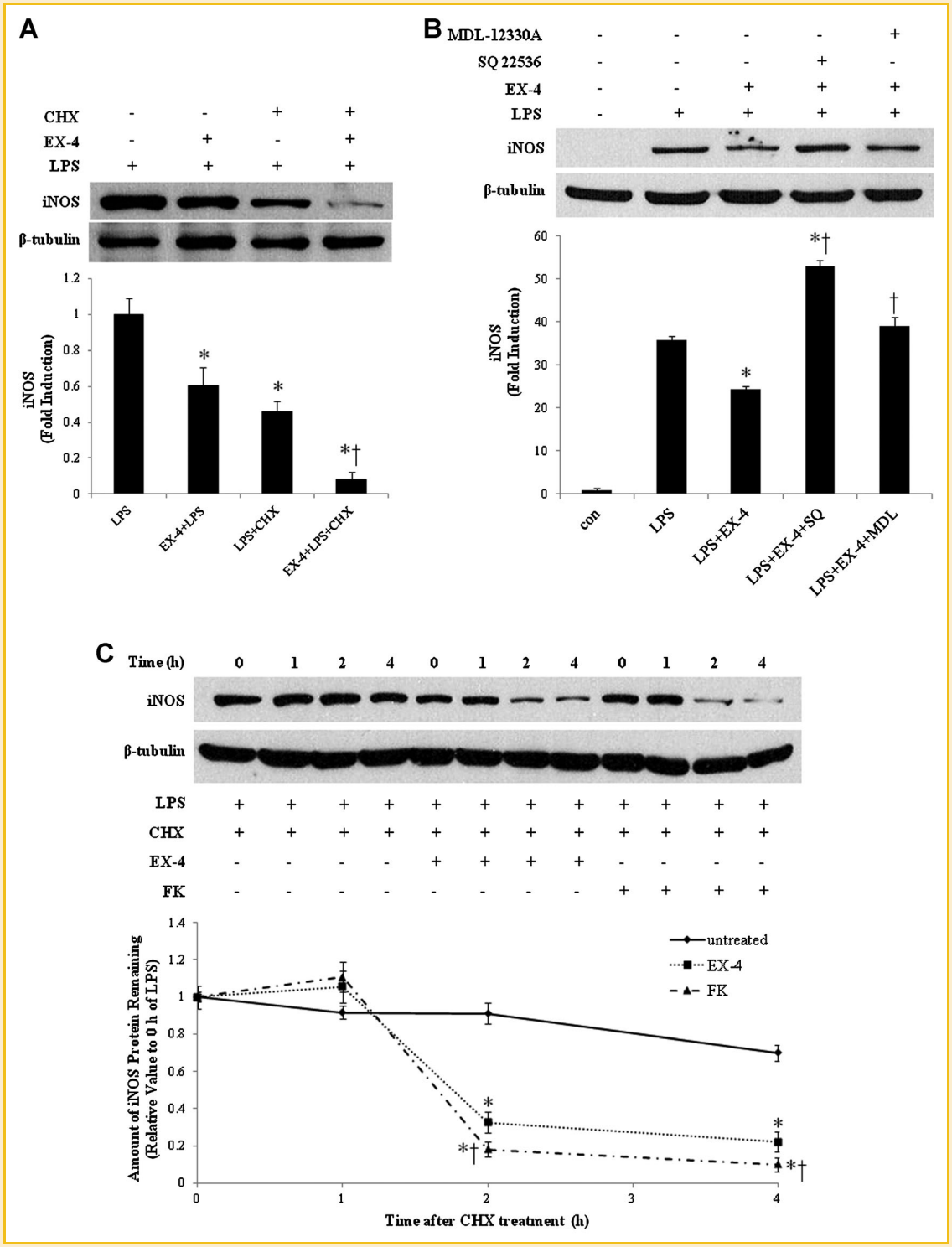

Fig. 5. The effect of exendin-4 on iNOS protein stability in Raw264.7 cells. A: The cells were pretreated with LPS (100 ng/ml) for 16 h, and then cycloheximide (CHX, 1 μg/ml) for 1 h to stop protein synthesis and next treated with exendin-4 (EX-4, 10 nM) for 4 h. Western blot analysis for iNOS was performed. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the LPS value. $^{*}P < 0.05$ versus LPS only; $^{\dagger}P < 0.05$ versus EX-4 + LPS. B: The cells were pretreated with LPS (100 ng/ml) for 16 h, and then treated with EX-4 (10 nM) or EX-4 and adenylate cyclase inhibitors MDL-12330A (5 μM) or SQ 22536 (10 μM) for 4 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 ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus LPS only; $^{\dagger}P < 0.05$ versus LPS + EX-4. C: The cells were pretreated with LPS (100 ng/ml) for 16 h, and then exposed to cycloheximide (CHX, 1 μg/ml) for 1 h to stop protein synthesis, and next treated with EX-4 (10 nM) or forskolin (FK, 10 μM) 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 plotted on amount of remaining protein relative to 0 h of LPS and represent the mean ± SD of four independent experiments. $^{*}P < 0.05$ versus untreated value; $^{\dagger}P < 0.05$ versus EX-4. D: The cells were pretreated with LPS (100 ng/ml) for 16 h, and then treated with EX-4 (10 nM) or EX-4 and H-89 (10 μM) for 6 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 ± SD of four independent experiments and each value is expressed relative to the control value. $^{*}P < 0.05$ versus LPS only; $^{\dagger}P < 0.05$ versus LPS + EX-4. E: Raw264.7 cells transfected with scramble siRNA (Scramble) or PKAα (PKA) siRNA were pretreated with LPS (100 ng/ml) for 16 h, and then treated with EX-4 (10 nM) for 6 h. Western blot analyzes for iNOS and PKA protein were performed. Equal loading of protein was verified by probing the same blot for β-tubulin. Data are expressed as mean ± SD of four independent experiments and each value is expressed relative to the scramble control value. $^{*}P < 0.05$ versus LPS + EX-4 in Scramble.

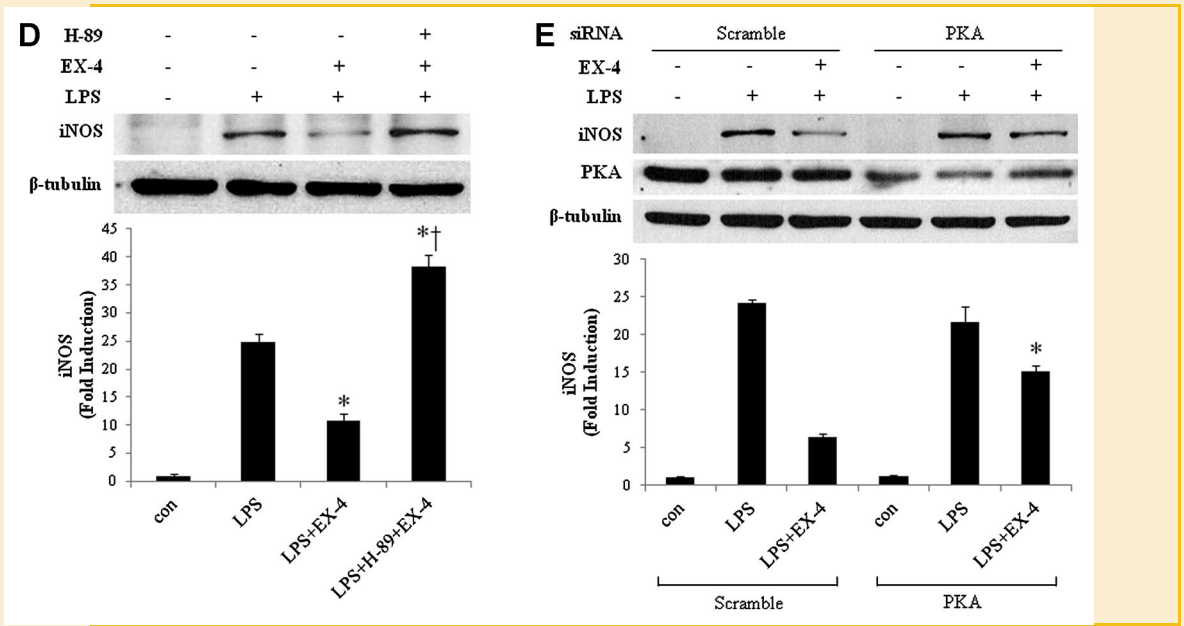

Fig. 5. (*Continued*)

expression in LPS-treated macrophages without inhibition of NF-κB activation [Paukkeri et al., 2007; Jin et al., 2009]. Therefore, our results indicate that the inhibitory effect of EX-4 on iNOS expression does not occur at the transcriptional level.

Besides the transcriptional mechanism, the posttranscriptional regulation is also involved in the induction of iNOS gene [Kleinert et al., 2004]. Therefore, to examine whether EX-4 inhibition of LPS-induced iNOS protein was attributable to the instability of iNOS mRNA, actinomycin D chase study was performed. EX-4 did not affect the degradation rate of iNOS mRNA induced by LPS. The 3′-untranslated region (UTR) of iNOS mRNA has AU-rich elements (ARE, AUUUA), which interact with ARE-binding proteins including HuR and polypyrimidine tract-binding protein. This regulatory *cis*-elements is known to play a role in modulating iNOS mRNA stability [Pautz et al., 2006]. Therefore, we analyzed the effect of EX-4 on iNOS mRNA stability using 3′-UTR-containing luciferase construct (pRiNOS-Luc-3′UTR). Consistent with the result of actinomycin D chase experiment, EX-4 did not inhibit the luciferase activity induced by LPS. Therefore, EX-4 may not have any significant effect on iNOS gene expression at the posttranscriptional level.

In addition to the transcriptional and posttranscriptional mechanisms, the posttranslational modulation of iNOS protein has been suggested [Kleinert et al., 2004]. Therefore, we analyzed the effects of EX-4 on iNOS protein stability using cycloheximide, a protein synthesis inhibitor. Interestingly, EX-4 significantly increased iNOS protein degradation, and cAMP increasing agent forskolin showed more potent effect than EX-4. To further explore the effects of EX-4 on iNOS protein expression, iNOS protein level was examined using adenylate cyclase inhibitor, PKA inhibitor and PKA gene silencing since the main effects of GLP-1 are mediated by the activation cAMP/PKA pathway [Brubaker and Drucker, 2004]. Our results showed that cAMP/PKA pathway was involved in the inhibitory effect of EX-4 on LPS-induced iNOS protein expression. These findings suggest that cAMP/PKA pathway is implicated in iNOS protein destabilization, which is supported by others' studies that cAMP enhanced tristetraprolin (TTP) protein degradation in LPS-treated J774 macrophages without altering TTP mRNA degradation rate [Jalonen et al., 2008] and EX-4 inhibited IL-1β-induced iNOS protein expression via cAMP/PKA system in INS-1 beta-cells [Kang et al., 2009], and also EX-4 reduced high glucose-induced thioredoxin interacting protein expression via cAMP/PKA signaling in INS-1 cells [Shao et al., 2010].

Meanwhile, the role of cAMP in iNOS expression has been controversial in macrophages: inhibitory [Marotta et al., 1992; Pang and Hoult, 1997] or stimulatory [Jeon et al., 1996]. However, much evidence supports that cAMP inhibits most inflammatory reactions such as the production of inflammatory cytokines and oxidative stress [Ottonello et al., 1995; Willis and Nisen, 1995]. In addition, EX-4 inhibited the expressions of inflammatory mediators TNF-α, monocyte chemoattractant protein-1 and IL-1β in macrophage cells [Arakawa et al., 2010; Kodera et al., 2011]. EX-4 also attenuated production of intercellular adhesion molecule-1 in TNF-α-stimulated glomerular endothelial cells [Kodera et al., 2011] and inhibited the expressions of iNOS and cyclooxygenase-2 mRNA in LPS-stimulated cardiomyoblasts [Chen et al., 2012]. It therefore seems likely that EX-4 acts as an anti-inflammatory agent by the activation of cAMP/PKA pathway.

Collectively, we found that EX-4 inhibited LPS-induced iNOS expression in Raw264.7 cells at the protein level without affecting the transcriptional and posttranscriptional mechanisms of iNOS gene expression, and this inhibitory effect of EX-4 was largely dependent on cAMP/PKA pathway. Thus, the effect of EX-4 on iNOS expression is at least a target for management of various inflammatory diseases.

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