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The role of nuclear factor-κB in the regulation of endothelin-1 production by nitric oxide

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Abstract

Nitric oxide (NO) has been reported to have an inhibitory effect on endothelin-1 production, but the detailed mechanisms are poorly understood. Our previous studies showed that a transcription factor nuclear factor-κB (NF-κB) plays an important role in endothelin-1 production. In the present study, we investigated the possible involvement of NF-κB in the inhibitory regulation of endothelin-1 production by NO. 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO), which is a well-known NO scavenger, remarkably increased both endothelin-1 production and NF-κB activation in cultured vascular endothelial cells. On the other hand, a spontaneous NO donor (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (FK409) significantly attenuated the basal and tumor necrosis factor-α-induced endothelin-1 production and NF-κB activation in endothelial cells. In addition, we found that FK409 suppressed NF-κB activation by the induction and stabilization of the inhibitory protein alpha, IκBα. Taken together, our results suggest that NO modulates the endothelial endothelin-1 production, possibly through the regulation of NF-κB activation.

Keywords: Nuclear factor-κB; Nitric oxide; Endothelin-1; Endothelial cell; Tumor necrosis factor-α

1. Introduction

Endothelin-1 is a potent vasoconstrictor peptide purified from the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988) and possesses a number of biological activities leading to vascular disorders (Miyauchi and Masaki, 1999). Endothelin-1 biosynthesis and release appear to be regulated at the transcriptional level because endothelin-1 release from endothelial cells is constitutive. Several studies have indicated that various substances such as thrombin (Kitazumi and Tasaka, 1993), transforming growth factor-β1 (Murata et al., 1995), and tumor necrosis factor (TNF)-α (Marsden and Brenner, 1992) stimulate the endothelin-1 gene expression in endothelial cells by DNA binding of transcription factors such as activator protein-1 and nuclear factor-1.

Nitric oxide (NO), known as an endothelium-derived relaxing factor, is formed from the terminal guanidino nitrogen atom of L-arginine by NO synthase (Moncada et al., 1991). NO influences a large variety of biological functions including vasodilatation, platelet aggregation, and neurotransmission (Moncada et al., 1991). In addition, it is well known that NO can reduce the endothelin-1 production in endothelial cells (Boulanger and Lüscher, 1990; Mitsutomi et al., 1999). However, it has not yet determined what mechanisms are responsible for the inhibitory effect of NO on endothelin-1 production.

Nuclear factor-κB (NF-κB) is a transcription factor with a pivotal role in inducing genes involved in physiological processes as well as in responses to injury and infection (Tak and Firestein, 2001). This transcription factor is found in the cytoplasm of most cells as an inactive complex bound to an inhibitory protein, IκB. It is well known that treatment of endothelial cells with a variety of stimuli including TNF-α results in the rapid activation of NF-κB, through the phosphorylation of IκB and its subsequent proteolytic degradation by the proteasome-dependent pathway (Tedgui and Mallat, 2001). We and others have recently reported that NF-κB is one of the modulators in endothelin-1 production (Quehenberger et al., 2000; Ohkita et al., 2002a,b).

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The purpose of the present study is to evaluate the possible involvement of NF-κB in the inhibitory regulation of endothelin-1 production by NO. To attain this, we investigated the effects of NO on both endothelin-1 production and NF-κB activation in cultured vascular endothelial cells using a NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and a spontaneous NO donor (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (FK409).

2. Materials and methods

2.1. Materials

Recombinant human TNF-α was purchased from Pepro Tech (Rocky Hill, NJ, USA). FK409 was a gift from Fujisawa Pharmaceutical (Osaka, Japan). Carboxy-PTIO was obtained from Dojindo Laboratories (Kumamoto, Japan). [^{125}I]Endothelin-1, [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). PSI [*N*-benzyloxycarbonyl-Ile-Glu (*O*-*t*-Bu)-Ala-leucinal] and BAY 11-7082 {(*E*)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile} were obtained from Peptide Institute (Osaka, Japan) and Calbiochem (San Diego, CA, USA), respectively. The culture reagents were purchased from Life Technologies (Grand Island, NY, USA). All other materials used were commercial products of the highest grade available.

2.2. Cell culture

Porcine aortic endothelial cells were isolated and were grown on gelatin-coated Petri dishes or plate in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 as described (Ohkita et al., 2002a). For all experiments, endothelial cells were grown to confluence then made quiescent by incubation with serum-free DMEM containing 0.01% heat-inactivated bovine serum albumin (Sigma, St. Louis, MO, USA) for 12 h.

2.3. Radioimmunoassay for determination of endothelin-1

The radioimmunoassay for endothelin-1 was performed as described (Ohkita et al., 2002a). Endothelin-1 antiserum was kindly provided by Dr. M.R. Brown, University of California, and did not cross-react with big endothelin-1 (Hexum et al., 1990).

2.4. Northern blot analysis

Total RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform extraction method. The isolated total RNA (5 μg per lane) was subjected to electrophoresis on a 1.1% agarose gel containing formaldehyde and transferred to Hybound-XL membrane (Amersham Pharmacia Biotech). This membrane was prehybridized for 1 h at 68 °C in PerfectHyb hybridization solution (Toyobo, Osaka, Japan) and then hybridized with porcine prepro endothelin-1 cDNA probe (a gift from Dr. K. Goto, University of Tsukuba, Japan) and GAPDH cDNA probe (Clontech laboratories, Palo Alto, CA, USA) labeled with [α-^{32}P]dCTP, using Random Primer DNA Labeling Kit (Takara Shuzo, Kyoto, Japan). After hybridization, the membrane was washed two times for 5 min followed by two more washes for 15 min at 68 °C in 2 × standard saline citrate/0.1% sodium dodecyl sulfate (SDS) (standard saline citrate contains 150 mM NaCl and

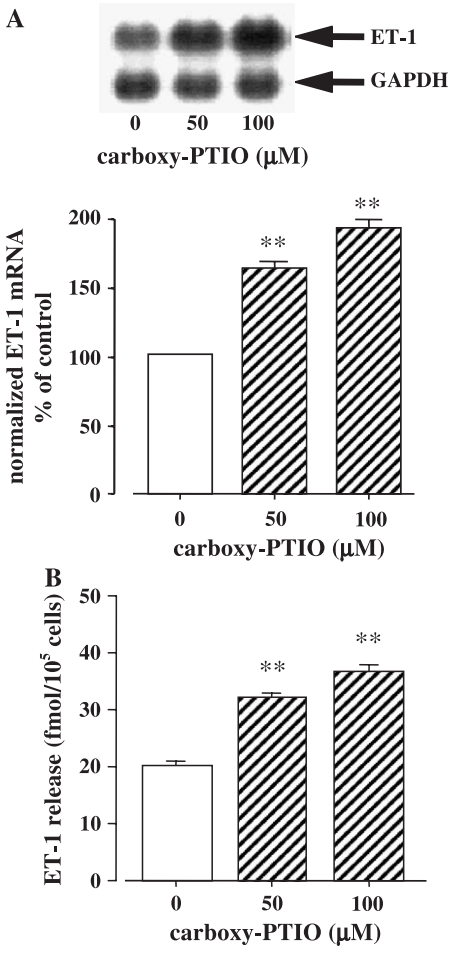

Fig. 1. Effects of carboxy-PTIO on endothelin-1 (ET-1) production in cultured porcine aortic endothelial cells. (A) Quiescent cells were treated with carboxy-PTIO (50 or 100 μM) for 4 h. Total cellular RNA (5 μg/lane) was analyzed using Northern blot analysis and hybridization with porcine prepro ET-1 and GAPDH cDNA used as probes. The signals for ET-1 mRNA were normalized to the corresponding GAPDH signals, quantified by densitometric analyses. Each value is expressed as the mean ± S.E.M. ($n=3$). **$P<0.01$, compared with control value. (B) Quiescent cells were incubated for 6 h in the absence or presence of carboxy-PTIO (50 or 100 μM), and the amount of ET-1 in the culture medium was measured using RIA. Each value is expressed as the mean ± S.E.M. ($n=8$). **$P<0.01$, compared with the control value.

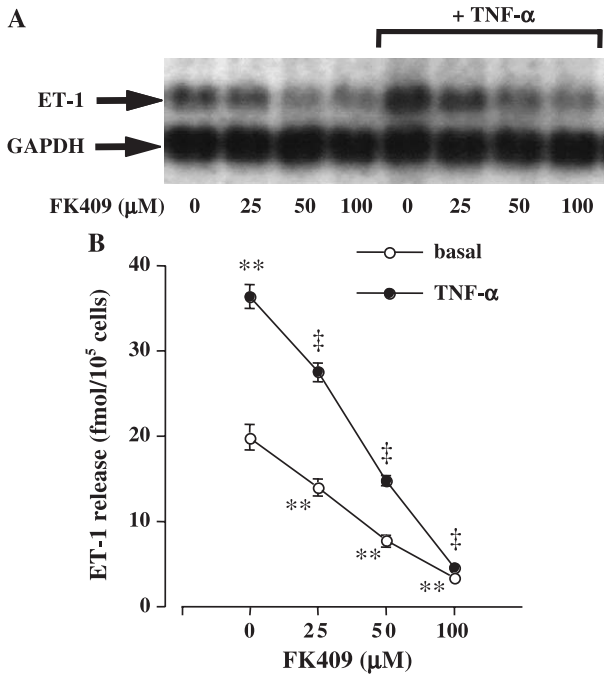

Fig. 2. Effects of FK409 on endothelin-1 (ET-1) production in cultured porcine aortic endothelial cells. (A) Quiescent cells were pretreated with FK409 (50 μM) for 5 min then stimulated with TNF-α (10 ng/ml) for 2 h. Total cellular RNA (5 μg/lane) was analyzed using Northern blot analysis and hybridization with porcine prepro ET-1 and GAPDH cDNA used as probes. (B) Quiescent cells were pretreated with FK409 for 5 min then stimulated with TNF-α (10 ng/ml). After stimulation with TNF-α for 6 h, the amount of ET-1 in the culture medium was measured. Each value is expressed as the mean ± S.E.M. ($n=6$). **$P<0.01$, compared with control value; ‡$P<0.01$, compared with TNF-α alone.

15 mM sodium citrate, pH 7.0). Autoradiography was performed by exposing the membrane to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA) at − 80 °C with intensifying screens. Autoradiograms of endothelin-1 were quantified by densitometric analyses, and signals of endothelin-1 mRNA were normalized for each sample, with respect to density of the corresponding signal for GAPDH mRNA.

2.5. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Endothelial cells were washed with ice-cold phosphate buffer saline and pelleted, then the cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), incubated for 15 min on ice, then lysed by the addition of 0.6% Nonidet P-40, followed by vigorous vortexing for 15 s. Nuclei were pelleted and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), and the tube vigorously shaken for 15 min at 4 °C. Nuclear extracts were then centrifuged , stored at − 80 °C, and used (2 μg protein) for EMSA. Double-stranded oligonucleotide containing the most common NF-κB consensus binding site 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega, Madison, WI, USA) was end-labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (Promega). The binding reaction was performed for 20 min at 25 °C in a total volume of 10 μl of binding buffer that contained 10 mM Tris−HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% glycerol, 0.5 μg of double stranded poly (dI-dC). DNA−protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 100 V for 2 h in 0.5 × Tris borate-EDTA (1 × Tris borate-EDTA which contains 90 mM Tris-borate, pH 8.3, 2 mM EDTA) at 4 °C. After electrophoresis, gels were dried and exposed to imaging plates (Fuji Film, Tokyo, Japan). The protein−DNA complexes were visualized using autoradiography.

2.6. Western blot analysis

All procedures were performed, according to the method described elsewhere (Ohkita et al., 2002b). Total protein (25 μg) was separated on a 12% SDS-polyacrylamide gel. After transfer to Hybond ECL nitrocellulose membranes (Amer-

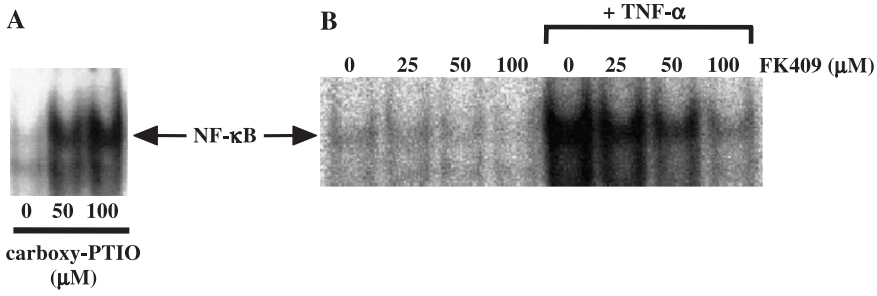

Fig. 3. Effects of carboxy-PTIO or FK409 on NF-κB activation in cultured porcine aortic endothelial cells. (A) Effect of carboxy-PTIO on basal NF-κB activation. Quiescent cells were incubated with the indicated concentrations of carboxy-PTIO for 2 h. NF-κB DNA binding activities were determined by EMSA. (B) Effects of FK409 on basal and TNF-α-induced NF-κB activation. Nuclear extracts were prepared from quiescent cells pretreated with the indicated concentrations of FK409 for 5 min then incubated with or without TNF-α (10 ng/ml) for 2 h. NF-κB DNA binding activities were determined by EMSA.

sham Pharmacia Biotech), the membranes were incubated overnight with rabbit polyclonal antibody for human IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature or rabbit polyclonal antibody for human phospho-IκBα (Ser32) (New England Biolabs, Beverly, MA, USA) at 4 °C. Membranes were washed and incubated with goat anti-rabbit horseradish peroxidase-linked immunoglobulin G (Zymed Laboratories, South San Francisco, CA, USA). Antibody-labeled proteins were detected by enhanced chemiluminescence Western blotting kits (Amersham Pharmacia Biotech).

2.7. Statistical analysis

All values are expressed as mean ± S.E.M. For statistical analysis, we used unpaired Student's *t*-test for two-group comparisons and one-way analysis of variance followed by Dunnett's multiple range tests for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of NO on endothelin-1 production in endothelial cells

As shown in Fig. 1A, when endothelial cells were incubated in the presence of carboxy-PTIO (50 and 100 μM), prepro endothelin-1 mRNA expression significantly increased in a dose-dependent manner (normalized prepro endothelin-1 mRNA level in 100 μM carboxy-PTIO-treated condition was about twofold of the control value). Fig. 1B shows the effect of carboxy-PTIO on endothelin-1 release from endothelial cells. Carboxy-PTIO (50 and 100 μM) increased endothelin-1 release by 60% and 80% from the control value of 20.2 ± 0.8 fmol/10^5 cells, respectively.

FK409 (25, 50, and 100 μM) markedly decreased basal and TNF-α-induced prepro endothelin-1 mRNA expression in endothelial cells (Fig. 2A). In addition, FK409 produced dose-related decreases in basal and TNF-α-induced endothelin-1 release from endothelial cells (Fig. 2B). At 100 μM, FK409 reduced endothelin-1 release to 17.3% and 12.4% of basal (19.8 ± 1.5 fmol/10^5 cells) and TNF-α-stimulated release (36.4 ± 1.5 fmol/10^5 cells), respectively.

Table 1
Effects of PSI and BAY 11-7082 on basal and TNF-α-induced endothelin-1 release from cultured porcine aortic endothelial cells

Treatments	Concentrations	Endothelin-1 release (%)
No addition		100 (5.44 ± 0.38 fmol/10^5 cells/h)
PSI	1 μM	77.18 ± 1.53[a]
BAY	5 μM	19.68 ± 0.60[b]
TNF-α	10 ng/ml	166.72 ± 8.26[a]
PSI + TNF-α	1 μM + 10 ng/ml	98.18 ± 4.17[c]
BAY + TNF-α	5 μM + 10 ng/ml	34.29 ± 5.95[c]

Each value is expressed as the mean ± S.E.M. ($n = 4–8$). [a]$P < 0.05$, [b]$P < 0.01$, compared with no addition. [c]$P < 0.01$, compared with TNF-α.

3.2. Involvement of NF-κB in the regulation of endothelin-1 production by NO

As shown in Fig. 3A, carboxy-PTIO markedly induced NF-κB activation under basal condition. On the other hand, FK409 attenuated the basal and TNF-α-stimulated NF-κB activation (Fig. 3B). To clarify which steps of NF-κB activation is regulated by NO, we examined the effects of FK409 on IκBα phosphorylation and degradation using Western blot analysis. As shown in Fig. 4, IκBα protein began to decrease 3 min after TNF-α addition, disappeared at 15 min and thereafter increased slightly and gradually. On the other hand, the amount of phosphorylated IκBα protein rapidly increased 3 min after TNF-α addition and disappeared gradually. Thereafter, the phosphorylated IκBα protein began to increase again. FK409 had no effects on IκBα phosphorylation and degradation after TNF-α addition, whereas this NO donor dose-dependently increased IκBα protein 30–90 min after TNF-α addition and almost completely restored the level of IκBα at 120 min.

3.3. Effects of PSI and BAY 11-7082 on endothelin-1 release from endothelial cells

In separate experiments, we evaluated the effect of inhibiting NF-κB activation on endothelin-1 production in

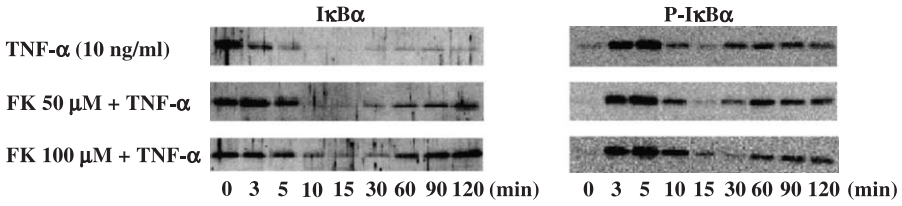

Fig. 4. Effects of FK409 on TNF-α-induced IκBα phosphorylation and degradation in cultured porcine aortic endothelial cells. Quiescent cells were pretreated with FK409 (50 or 100 μM) for 5 min and then incubated with or without TNF-α (10 ng/ml) for the indicated times. The cell lysate was subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against IκBα (left panel) and phosphorylated IκBα (right panel).

endothelial cells. As shown in Table 1, PSI, a proteasome inhibitor, and BAY 11-7082, an inhibitor of NF-κB, significantly suppressed both basal and TNF-α-induced endothelin-1 release.

4. Discussion

It has been shown that endogenous NO is capable of reducing endothelin-1 production in endothelial cells, based on findings indicating that an inhibition of endogenous NO biosynthesis with NO synthase inhibitors efficiently increases the endothelial endothelin-1 production (Boulanger and Lüscher, 1990; Cao et al., 1994; Mitsutomi et al., 1999). In addition, an exogenous NO is reported to have an inhibitory effect on endothelin-1 secretion and its mRNA expression in endothelial cells (Takada et al., 1996; Smith et al., 2002). Thus, it seems likely that both endogenous and exogenous NO play an important role in the regulation of endothelin-1 production, but it remains obscure how NO can affect the endothelin-1 production in endothelial cells. In the present study, we noted that elimination of endogenous NO with a NO scavenger carboxy-PTIO resulted in a significant up-regulation of basal prepro endothelin-1 mRNA expression in endothelial cells. This potentiating effect of carboxy-PTIO was accompanied by an increase in endothelin-1 release from endothelial cells under basal condition. On the other hand, we observed that a spontaneous NO donor FK409 markedly suppressed basal and TNF-α-induced prepro endothelin-1 mRNA expression and the subsequent endothelin-1 release. Taken together, both carboxy-PTIO and FK409 seem to affect the endothelin-1 production at its transcriptional level.

It is well known that transcriptional factors such as activator protein-1, GATA-2, and nuclear factor-1 play an important role in the regulation of transcriptional activity of endothelin-1 gene (Kawana et al., 1995; Miyauchi and Masaki, 1999). Most recently, there is accumulating evidence indicating that NF-κB is also responsible for the regulation of endothelin-1 gene expression in endothelial cells. This view is based on findings that various NF-κB suppressors such as proteasome inhibitors, antioxidants, and an inhibitor of IκBα phosphorylation can decrease endothelin-1 production in endothelial cells (Ohkita et al., 2002a,b). Also in the present study, we observed that a proteasome inhibitor PSI efficiently suppressed both basal and TNF-α-induced endothelin-1 release from endothelial cells. The proteasome-dependent proteolytic pathway is known to be involved in the activation process of NF-κB (Palombella et al., 1994; Traenckner et al., 1994). Qualitatively similar effect was obtained by using BAY 11-7082, an inhibitor of NF-κB, which prevents a step of the phosphorylation of IκBα (Pierce et al., 1997). Recently, Quehenberger et al. (2000) found that the κB site, a binding sequence for the activated NF-κB, is located in the promoter region of human endothelin-1 gene. In the present study, we demonstrated that carboxy-PTIO remarkably induced the activation of NF-κB under basal conditions, whereas FK409 had an opposite effect. Furthermore, FK409 could dose-dependently inhibit TNF-α-induced NF-κB activation in endothelial cells. Thus, it is reasonable to consider that changes of NF-κB activation contribute to the effects of carboxy-PTIO and FK409 on endothelin-1 gene expression and the subsequent endothelin-1 release in basal and TNF-α-stimulated conditions.

Various cytokines such as TNF-α and interleukin-1 have been demonstrated to increase the production of reactive oxygen species (Bowie and O'Neill, 2000). It has been reported also that reactive oxygen species play an important role in the step of IκBα phosphorylation (Ginn-Pease and Whisler, 1998). Thus, one can speculate that NO has an inhibitory effect on the phosphorylation of IκBα by trapping reactive oxygen species; however, the present experiment using FK409 clearly indicated that this NO donor did not affect the transient decrease in IκBα levels as well as its rapid phosphorylation induced by TNF-α, whereas FK409 rapidly increased the expression of IκBα protein as early as 30 min after the drug addition and almost completely restored to the control level at 120 min. These findings suggest that the inhibitory effect of FK409 on NF-κB activation is due to the induction and stabilization of IκBα. Peng et al. (1995) have reported that a NO donor *S*-nitrosoglutathione (GSNO) prevented TNF-α-induced NF-κB activation by induction and stabilization of IκBα in human endothelial cells. Furthermore, they have demonstrated that GSNO inhibited TNF-α-induced NF-κB activation and its nuclear translocation, accompanied by the induction of both IκBα mRNA and protein expression (Spiecker et al., 1997). Taken together with our results, the mechanism by which FK409 increases the IκBα protein level is probably attributed to the enhanced IκBα gene expression by NO, although further experiments are required to clarify whether FK409 can induce IκBα gene expression. On the other hand, FK409 did not have any inhibitory effects on TNF-α-induced IκBα phosphorylation as mentioned above. Katsuyama et al. (1998) demonstrated that FK409 inhibited interleukin-1-induced NF-κB activation in vascular smooth muscle cells by interfering with IκBα phosphorylation rather than the induction and stabilization of IκBα. The reason for this discrepancy is unclear, but these contradictory results may be related to the differences in experimental methods and materials (e.g., cell types, species, cytokines, and pretreatment time of NO donors).

In conclusion, both endogenous and exogenous NO have an inhibitory effect on endothelial endothelin-1 production at transcriptional level through the regulation of NF-κB activation. In addition, it seems likely that the induction and/or stabilization of IκBα by NO are closely related to the above inhibitory regulation.

Acknowledgements

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