Nitric oxide inhibits endothelin-1 production through the suppression of nuclear factor κB

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ABSTRACT
We have reported previously that the nitric oxide (NO) donor FK409 \((\pm)-(E)-4\text{-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexanamide}\) improved renal dysfunction as well as renal lesions in rats with ischaemia/reperfusion injury. We also found that FK409 substantially reduced endothelin-1 (ET-1) production in cultured vascular endothelial cells (ECs). Nuclear factor κB (NF-κB) is known to play a key role in the development of ischaemic disorders through regulation of the expression of a variety of genes. In the present study, we examined the effects of FK409 on ET-1 production in cultured pig aortic ECs, and the possible involvement of NF-κB in the inhibitory effect of NO on ET-1 production. FK409 significantly attenuated basal and tumour necrosis factor-α (TNF-α)-induced preproET-1 mRNA expression in ECs. In addition, FK409 diminished basal and TNF-α-stimulated NF-κB activation in ECs. Pretreatment with \(N\)-benzyloxycarbonyl-Ile-Glu(O-t-Bu)-Ala-leucinal or BAY 11-7082, both of which are suppressors of NF-κB activation, effectively attenuated basal and TNF-α-induced ET-1 mRNA expression. These findings suggest that the suppression of NF-κB activation is at least partly involved in the FK409-induced inhibition of ET-1 production in ECs. We propose that NF-κB activation plays an important role in ET-1 production.

INTRODUCTION
It is well known that endothelin-1 (ET-1) increases nitric oxide (NO) release from endothelial cells (ECs) through stimulation of the endothelin ET\(_B\) receptor [1]. On the other hand, there are several reports on the inhibitory regulation of ET-1 production by NO in ECs [2,3], but the mechanism underlying this inhibition remains unclear.

The transcription factor nuclear factor κB (NF-κB) plays an important role in the development and maintenance of various vascular diseases, such as atherosclerosis and ischaemia/reperfusion injury, based on evidence showing that NF-κB controls the expression of a variety of genes, including those encoding cytokines and adhesion molecules [4]. Therefore the inhibition of NF-κB activation may be useful in the treatment of such vascular diseases.

We reported previously that the NO donor FK409 \((\pm)-(E)-4\text{-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexanamide}\) could improve renal dysfunction as well as renal lesions in rats with ischaemia/reperfusion injury [5]. We also found that FK409 substantially reduced ET-1 production in cultured vascular ECs [6]. The present study was designed to investigate the inhibitory effect of FK409 on ET-1 production. We now report that the inhibition of ET-1 production by NO is due to the suppression of NF-κB activation.

**Key words:** endothelial cells, endothelin-1, NF-κB, nitric oxide, nitric oxide donor.

**Abbreviations:** EC, endothelial cell; ET-1, endothelin-1; NF-κB, nuclear factor κB; PSI, \(N\)-benzyloxycarbonyl-Ile-Glu(O-t-Bu)-Ala-leucinal; TNF-α, tumour necrosis factor-α.

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MATERIALS AND METHODS

Cell culture
Pig aortic ECs were isolated, and were grown on gelatin-coated Petri dishes or plates in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air/5% CO₂ as described in [6]. All cell cultures were used from passages 5 to 8. For all experiments, ECs were grown to confluence and then made quiescent by incubation with serum-free Dulbecco’s modified Eagle’s containing 0.01% heat-inactivated BSA for 12 h.

Northern blot analysis
All procedures were carried out according to methods described elsewhere [6]. 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% (w/v) agarose gel containing formaldehyde, and transferred to a nylon membrane. This membrane was hybridized with cDNA probes for pig preproET-1 and glyceraldehyde-3-phosphate dehydrogenase labelled with [α-³²P]dCTP. After hybridization, the membrane was washed and exposed to X-ray film at −80 °C.

Preparation of nuclear extracts and electrophoretic mobility shift assay
Nuclear extracts were prepared from ECs using the method of Schreiber et al. [7]. The nuclear extracts (2 μg of protein) were used for electrophoretic mobility shift assays. A double-stranded oligonucleotide containing the most common NF-κB consensus binding site, 5’-AGT TGA GGG GAC TTT CCC AGG C-3’, was end-labelled with [γ-³²P]ATP, using T4 polynucleotide kinase. DNA–protein complexes were resolved on non-denaturing 6% (w/v) polyacrylamide gels. After electrophoresis, gels were dried and exposed to X-ray film at −80 °C.

Statistical analysis
All values are expressed as means ± S.E.M. For statistical analysis, we used one-way ANOVA followed by Bonferroni’s multiple comparison tests. Differences were considered statistically significant at P < 0.05.

RESULTS

Effects of FK409 on expression of preproET-1 mRNA and NF-κB activation
The effects of FK409 on preproET-1 mRNA expression were examined using Northern blot analysis. When ECs were incubated in the presence of tumour necrosis factor-α (TNF-α; 10 ng/ml), expression of preproET-1 mRNA increased significantly (normalized preproET-1 mRNA level in TNF-α-stimulated conditions was about 1.9-fold of the control value). FK409 (50 μM) reduced basal and TNF-α-induced preproET-1 mRNA expression (Figures 1A and 1B). FK409 also inhibited TNF-α-induced NF-κB activation (Figure 1C).

Effects of NF-κB suppressors on expression of preproET-1 mRNA
To evaluate the possible involvement of NF-κB in the inhibition of ET-1 production by NO, we investigated the effects of NF-κB suppressors on preproET-1 mRNA expression. As shown in Figure 2, N-benzyloxycarbonyl-Ile-Glu(O-t-Bu)-Ala-leucinal (PSI) and BAY 11-7082, both of which are suppressors of NF-κB activation [8,9],...
effectively decreased basal and TNF-α-induced preproET-1 mRNA expression.

**DISCUSSION**

It is well known that a balance between NO and ET-1 production in ECs plays a central role in maintenance of the integrity of vascular tone. NO is thought to cause relaxation of vascular smooth muscle by activation of soluble guanylate cyclase and elevation of intracellular cGMP [10]. However, there is evidence that NO acts via mechanisms that do not involve the guanylate cyclase/cGMP pathway, such as activation of potassium channels [11] or Na+/K+ ATPase [12] on the cell membrane. Our previous study [6] showed that endogenous NO appears to suppress ET-1 production, via guanylate cyclase/cGMP-dependent mechanisms. On the other hand, we noted that exogenously applied NO could suppress ET-1 production at the transcriptional level, possibly through a guanylate cyclase/cGMP-independent pathway. However, the mechanisms that play an important role in the inhibitory effect of exogenous NO on ET-1 gene expression remain to be elucidated.

NF-κB is a transcription factor that has a pivotal role in inducing genes involved in physiological processes, as well as in the response to injury and infection. 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 ECs 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 proteolytic pathway [4].

NO has been reported to inhibit NF-κB activation, and two possible mechanisms for this inhibition are proposed. First, NO can suppress NF-κB activation by inducing and stabilizing IκB [13], as well as by interfering with IκB phosphorylation [14]. Secondly, NO can interfere directly with the binding of NF-κB to its response element, by nitrosylation of Cys^62 of the p50 protein [15]. In present study, we also noted that a NO donor, FK409, suppressed NF-κB activation in ECs. In addition, we have also observed that the suppressive effect of FK409 is due to the induction and/or stabilization of IκB (results not shown). To determine whether FK409 inhibits ET-1 gene expression through the inhibition of NF-κB activation, we examined the effects of an IκB kinase inhibitor (BAY 11-7082) and a proteasome inhibitor (PSI) on preproET-1 mRNA expression. Our results showed that both BAY 11-7082 and PSI efficiently decreased basal and TNF-α-induced preproET-1 mRNA expression in ECs. While the present study was in progress, Quehenberger et al. [16] demonstrated that the human ET-1 gene has an NF-κB binding site, and confirmed that transcription of the ET-1 gene is controlled by NF-κB. Taken together, it seems likely that the inhibitory effects of FK409 on ET-1 production are attributable, at least in part, to the suppression of NF-κB activation. Finally, we propose that NF-κB activation plays an important role in ET-1 production.

**REFERENCES**


