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A Nuclear Factor-κB Inhibitor BAY 11-7082 Suppresses Endothelin-1 Production in Cultured Vascular Endothelial Cells

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ABSTRACT—BAY 11-7082, an inhibitor of nuclear factor-κB (NF-κB), which prevents a step of the phosphorylation of inhibitory protein IκB bound to NF-κB, suppressed basal and tumor necrosis factor (TNF)-α-induced prepro endothelin (ET)-1 mRNA expression and NF-κB activation in cultured vascular endothelial cells. BAY 11-7082 significantly decreased basal and TNF-α-induced ET-1 release from endothelial cells. These results indicate that the inhibition of NF-κB activation contributes to the suppressive effect of BAY 11-7082 on ET-1 gene expression and ET-1 release, thereby suggesting that NF-κB plays an important role in the regulation of ET-1 production.

Keywords: Nuclear factor-κB, Endothelin-1, Tumor necrosis factor-α

Endothelin (ET)-1 is a potent vasoconstrictive peptide purified from the supernatant of cultured porcine aortic endothelial cells (ECs) (1). ET-1 biosynthesis and release appear to be regulated at the transcriptional level because ET-1 release from ECs is constitutive. It is well known that various substances such as thrombin, transforming growth factor-β1 and tumor necrosis factor (TNF)-α increase the transcription of ET-1 gene (2), but the regulatory mechanisms of ET-1 production remain poorly understood.

Nuclear factor-κB (NF-κB) is an inducible transcription factor that is primarily involved in immune, inflammatory and stress responses (3). This transcription factor is found in the cytoplasm of most cells as an inactive complex bound to an inhibitory protein, IκB. It has been shown that treatment with a variety of stimuli including cytokines and oxidative stress results in the activation of NF-κB, through the phosphorylation of IκB and its subsequent proteolytic degradation by the proteasome-dependent proteolytic pathway (4). The activated NF-κB translocates to the nucleus to enhance the transcription of many inflammatory genes such as cytokines, chemokines and adhesion molecules (4, 5). Therefore, it has been suggested that prevention of NF-κB activation is a novel approach to the treatment of several vascular diseases (5).

The purpose of the present study is to evaluate the effectiveness of selective prevention of NF-κB activation on endothelial ET-1 production. To attain this, we investigated the effect of a IκBα phosphorylation inhibitor BAY 11-7082 {(*E*)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile) (6) on basal and TNF-α-induced ET-1 production in cultured vascular ECs.

Porcine aortic ECs 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. For all experiments, ECs were grown to confluence and then made quiescent by incubation with serum-free DMEM containing 0.01% heat-inactivated bovine serum albumin for 12 h.

The radioimmunoassay (RIA) for ET-1 was done as described (7). ET-1 antiserum was kindly provided by Dr. M.R. Brown, University of California and did not cross-react with big ET-1 (8).

For Northern blot analysis, the isolated total RNA (5 μg per lane) was subjected to electrophoresis on a 1.1% agarose gel containing formaldehyde and transferred to a nylon membrane. This membrane was hybridized with porcine prepro ET-1 cDNA probe (a gift from Dr. K. Goto, University of Tsukuba) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA, USA) labeled with [α-^{32}P]dCTP. After hybridization, the membrane was exposed to X-ray films at −80°C. The autoradiograms of ET-1 were quantified by densitometric analyses, and signals of ET-1 mRNA were normalized for each sample, with respect to density of the corresponding signal for GAPDH mRNA.

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Electrophoretic mobility shift assay (EMSA) was done, according to the method described elsewhere (7). 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. DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. After electrophoresis, gels were dried and exposed to imaging plates (Fuji Film, Tokyo) at room temperature. The protein-DNA complexes were visualized using autoradiography.

For Western blot analysis, total protein (25 μg) was separated on a 12% SDS-polyacrylamide gel. After transfer to nitrocellulose membranes, 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 IgG (Zymed Laboratories, South San Francisco, CA, USA). Antibody-labeled proteins were detected by enhanced chemiluminescence Western blotting kits (Amersham, Arlington Heights, IL, USA).

All values are expressed as mean ± S.E.M. For statistical analyses, we used the 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 $P<0.05$.

The pretreatment of ECs with BAY 11-7082 (Calbiochem, San Diego, CA, USA) caused a dose-dependent decrease in TNF-α-induced NF-κB activation (Fig. 1A). Although the changes in NF-κB activation by BAY 11-7082 in basal conditions was not easily viewable, the suppressive effect of BAY 11-7082 on basal NF-κB activation was detectable when gels were exposed to imaging plates for longer periods. Figure 1B shows the effects of TNF-α on the dynamics of IκBα and phosphorylated IκBα protein in ECs. The IκBα protein content began to decrease after 5 min of TNF-α addition, disappeared at 15 min and then increased gradually. On the other hand, the amount of phosphorylated IκBα protein rapidly increased after 5 min of TNF-α addition and disappeared at 15 min. Thereafter, the phosphorylated IκBα protein content began to increase again, as well as did IκBα. These results show that IκBα is rapidly phosphorylated, followed by a degradation during the activation of NF-κB by TNF-α. The pretreatment with BAY 11-7082 stabilized the amount of IκBα protein in ECs by the suppression of TNF-α-induced IκBα phosphorylation (Fig. 1C). These results indicate that BAY 11-7082 selectively suppresses the phosphorylation of IκBα rather than the degradation of phosphorylated IκBα.

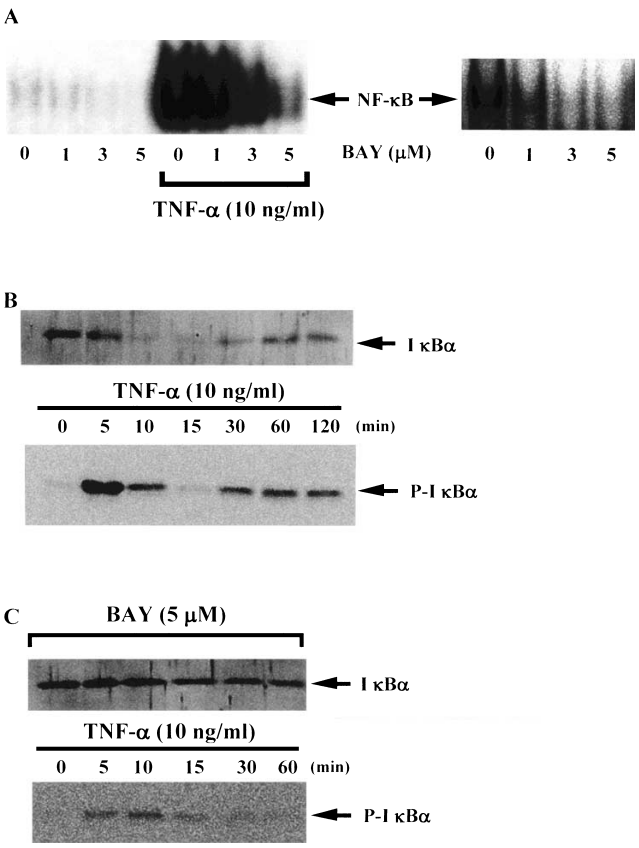

Fig. 1. Effects of BAY 11-7082 on NF-κB activation pathway. A: Effects of BAY 11-7082 on basal and TNF-α-induced NF-κB activation (left panel). Nuclear extracts were prepared from quiescent ECs pretreated with the indicated concentrations of BAY 11-7082 for 1 h and then incubated with or without TNF-α (10 ng/ml) for 15 min in the continued presence of BAY 11-7082. NF-κB DNA binding activities were determined by EMSA. Effect of BAY 11-7082 on basal NF-κB activation was determined by a long-time autoradiography (right panel). B: Effects of TNF-α on IκBα phosphorylation and degradation in ECs. Quiescent ECs were stimulated with TNF-α (10 ng/ml) for the indicated times. The cells were then lysed, and the cell lysate (25 μg protein) was subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against IκBα (upper panel) and phosphorylated IκBα (lower panel). C: Effects of BAY 11-7082 on TNF-α-induced IκBα phosphorylation and degradation in ECs. Quiescent ECs were pretreated with BAY 11-7082 (5 μM) for 1 h and then incubated with or without TNF-α (10 ng/ml) for the indicated times in the continued presence of BAY 11-7082. The cell lysate was subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against IκBα (upper panel) and phosphorylated IκBα (lower panel).

As shown in Fig. 2, A and B, BAY 11-7082 decreased basal and TNF-α-induced prepro ET-1 mRNA expression in ECs. At the highest concentration of BAY 11-7082 (5 μM), both basal and TNF-α-induced prepro ET-1 mRNA expression were markedly suppressed in a time-dependent manner (Fig. 2C). In addition, BAY 11-7082 produced dose-related decreases in basal and TNF-α-

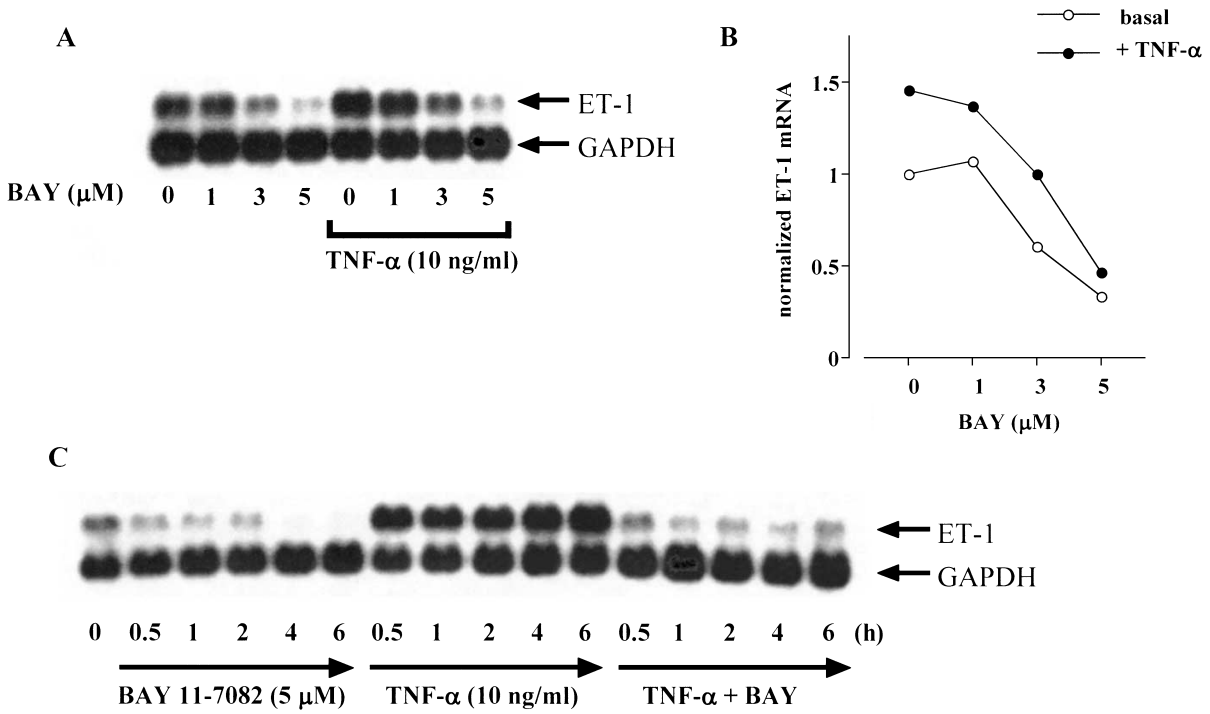

Fig. 2. Effects of BAY 11-7082 on basal and TNF-α-induced prepro ET-1 mRNA expression in ECs. A and B: Quiescent ECs were pretreated with the indicated concentrations of BAY 11-7082 for 1 h, and then they were stimulated with or without TNF-α (10 ng/ml) for 4 h in the continued presence of BAY 11-7082. To normalize signals for prepro ET-1 mRNA, GAPDH mRNA levels were compared as an internal control. Results are expressed as the mean of two experiments. C: Quiescent ECs were pretreated with BAY 11-7082 (5 μM) for 1 h, and then they were stimulated with or without TNF-α (10 ng/ml) for the indicated times in the continued presence of BAY 11-7082. Prepro ET-1 mRNA and GAPDH mRNA levels were measured by Northern blot analysis.

induced ET-1 release from ECs: BAY 11-7082 (5 μM) reduced the ET-1 release to 19.0% and 19.1% of basal (30.6 ± 2.4 fmol/10^5 cells) and TNF-α-stimulated release (52.4 ± 1.8 fmol/10^5 cells), respectively (Fig. 3).

BAY 11-7082 was synthesized as a novel inhibitor of NF-κB activation and was shown to suppress IκBα phosphorylation (6). In the present study, we found that BAY 11-7082 had a potent suppressive effect on basal and TNF-α-induced NF-κB activation in ECs. These findings indicate that BAY 11-7082 is a useful tool for clarifying physiological and pathophysiological roles of NF-κB.

It is well known that transcriptional factors such as activator protein-1, GATA-2 and nuclear factor-1 predominantly play an important role in the regulation of transcriptional activity of ET-1 gene (2). However, it remains unclear which type of transcription factors is responsible for the TNF-α-induced ET-1 gene expression in vascular ECs. The present study showed that BAY 11-7082 attenuated the basal and TNF-α-induced prepro ET-1 mRNA expression in a dose-dependent manner. In addition, ET-1 release from ECs was markedly suppressed by BAY 11-7082 both in basal and TNF-α-stimulated conditions. Thus, it is most likely that the suppression of NF-κB

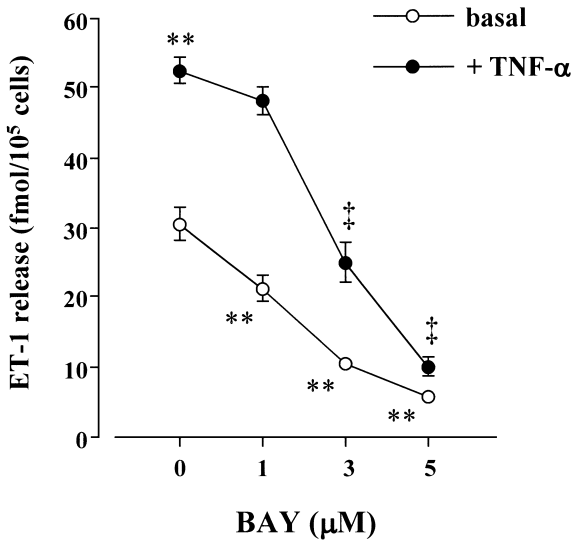

Fig. 3. Effects of BAY 11-7082 on ET-1 release from porcine aortic ECs. Quiescent ECs were pretreated for 1 h with the indicated concentrations of BAY 11-7082 followed by 6 h incubation with or without TNF-α (10 ng/ml) in the continued presence of BAY 11-7082. Results are expressed as the mean ± S.E.M. (n = 6). **P<0.01 versus no addition. $^{‡}P$<0.01 versus TNF-α alone.

activation is involved in the mechanism for preventive effects of BAY 11-7082 on prepro ET-1 mRNA expression and the consequent decrease in ET-1 release. Recently, Quehenberger et al. have demonstrated that human ET-1 gene has an NF-κB binding site sequence and confirmed that transcription of ET-1 gene is controlled by NF-κB in advanced glycation end product-stimulated cultured ECs (9). Taken together, it is reasonable to consider that NF-κB functions as one of main regulatory factors both in basal and TNF-α-induced ET-1 gene transcription.

In the present study, although BAY 11-7082 had little effect on basal prepro ET-1 mRNA expression at 1 μM, the basal ET-1 release from EC was significantly reduced by BAY 11-7082 at the same concentration. The reason for this is unclear, but it has been shown that ET-1 production can be regulated by post-transcriptional as well as transcriptional mechanisms. Thus, one possible explanation is that BAY 11-7082 may influence the ET-1 mRNA expression at the post-transcriptional mechanisms such as the stabilization of ET-1 mRNA.

As well as BAY 11-7082, antioxidants and proteasome inhibitors are well known to prevent the activation of NF-κB: antioxidants inhibit a step of IκB phosphorylation (10), and proteasome inhibitors suppress the degradation of phosphorylated IκB (11). We previously noted that these drugs attenuated ET-1 production, both in vivo (12 – 14) and in vitro (7). Our findings suggested that the suppressive effect of antioxidants or proteasome inhibitors on ET-1 production might be due to the inhibition of NF-κB activation. However, it remained obscure, because these drugs exhibit not only the suppression of NF-κB but also extensive influences on various intracellular events (11, 15). In the present study, we found that the selective inhibition of IκBα phosphorylation in the activation process of NF-κB resulted in the reduction of ET-1 production in ECs. Taken together, it is conceivable that the reduction of ET-1 production with antioxidants or proteasome inhibitors results from the prevention of NF-κB activation, and that drugs, which can inhibit any step in NF-κB activation, may suppress the ET-1 production.

In conclusion, our results clearly indicate that prevention of NF-κB activation reduces endothelial ET-1 production at transcriptional level, and that NF-κB functions as one of the primary signals in ET-1 production. Furthermore, findings from the present study suggest that selective suppression of NF-κB activation may also be a pertinent treatment of various vascular diseases such as atherosclerosis and ischemia/reperfusion with aberrant ET-1 production or NF-κB activation.

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