

Carvedilol Inhibits Expressions of Vascular Cell Adhesion Molecule-1, Intercellular Adhesion Molecule-1, Monocyte Chemoattractant-1, and Interleukin-8 via NF- κ B Inhibition in Human Endothelial Cells

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ABSTRACT

Background and Objectives : Carvedilol is an anti-oxidative, the cardioprotective effects of which are mediated by the inhibition of NF- κ B activation. The present study was designed to examine the effects of carvedilol, an α 1- and β -blocker, on tumor necrosis factor (TNF)- α stimulated human umbilical vein endothelial cells (HUVEC). **Materials and Methods :** HUVEC were treated with TNF- α (10 ng/mL) in either the absence or presence of carvedilol. The levels of intracellular reactive oxygen species (ROS) were examined using a fluorescent dye DCFH-DA, with the adhesion of U-937 monocyte to the HUVEC. Nuclear factor kappa B (NF- κ B) activation was determined by NF- κ B p65 translocation to the nucleus using Western blotting and immunocytochemistry. The expressions of NF- κ B dependent pro-inflammatory molecules, i.e., vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8, were measured by RT-PCR and ELISA. Bcl-2 and phosphorylation of c-Jun N-terminal protein kinase (JNK) were measured using Western blotting. **Results :** TNF- α treatment increased the activation of NF- κ B, suppressed Bcl-2, and increased the phosphorylation of JNK, the ROS level and the adhesion of U-937. The levels of mRNA and protein expressions of VCAM-1, ICAM-1, MCP-1 and IL-8 were up-regulated by TNF- α . Carvedilol inhibited the phosphorylation of JNK, ROS formation and the adhesion of U-937 monocyte. In addition, carvedilol reduced the production of VCAM-1, ICAM-1, MCP-1 and IL-8 at the mRNA and protein levels, via the suppression of NF- κ B activation. **Conclusion :** These results suggested that the anti-inflammatory effects of carvedilol on TNF- α stimulated endothelial cells could be explained by its ROS-scavenging and NF- κ B inactivation properties. (Korean Circulation J 2005;35:576-582)

KEY WORDS : Carvedilol ; Tumor necrosis factor- α , nuclear factor- κ B, endothelial cell.

Introduction

Inflammation has been demonstrated to play an important role in the formation and progression of atherosclerosis.^{1,2)} TNF- α is one of the major inflammatory mediators that promotes the pathogenesis of

atherosclerosis, and is present in all stages of atherosclerosis.³⁾ TNF- α enhanced the expressions of the adhesion molecules on the membrane of endothelial cells,⁴⁾ transcription factors related with inflammation, such as NF- κ B, and caused modulation of the activities of various enzymes, such as mitogen-activated protein kinases (MAPK).^{5,6)} In various pathological conditions, such as ischemia and acute inflammation, excessively accumulated reactive oxygen species (ROS) promote the activations of MAPKs or apoptosis cascades.⁵⁾

Carvedilol, a vasodilating and non-selective α 1- and β -adrenoreceptor antagonist, is currently used for the treatment of hypertension and heart failure. Besides its anti-hypertensive effects, many reports have demon-

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strated that carvedilol reduced infarct sizes and prevented restenosis after angioplasty and coronary atherectomy.^{7,9)} Carvedilol has also been reported to act as an anti-oxidative *in vitro*, with potency 30 to 80 times higher than vitamin E or probucol.¹⁰⁾

The aim of the present study was to investigate the effects of carvedilol on TNF- α -induced cytokine expressions and NF- κ B activation. The involvement of NF- κ B activation was confirmed using BAY 11-7082, a specific inhibitor of NF- κ B.

Materials and Methods

Materials

The carvedilol was kindly provided by Chong Kun Dang Pharm. The BAY 11-7082 was purchased from Biomol (Plymouth Meeting, PA) and the TNF- α from R & D Systems (Minneapolis, MN). The BCA protein assay kit was from Bio-Rad Laboratories (Hercules, CA). The VCAM-1, human α -actin, Bcl-2 and NF- κ B p65 antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). The phosphorylated JNK antibody was from Cell Signaling (Beverly, MA). The reverse transcriptase, Taq DNA polymerase, NuPAGE Pre-Cast Gels and Western Breeze kits were from Invitrogen (Carlsbad, CA). The DuoSet ELISA development kit for human IL-8 was from R & D System. The NE-PER nuclear and cytoplasmic extraction reagents were from Pierce Biotechnology (Rockford, IL), the HUVEC from Cloneteck (San Diego, CA), and the RBMI1640 and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY). The endothelial basal medium (EBM-2) and Single Quot kit were from Cloneteck, and the 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were from Sigma (St. Louis, MO). Apparatus for the cell cultures were from Nunc (Rochester, NY).

Cell culture

HUVEC (passage 4-6) were maintained in EBM-2, supplemented with Single Quot kit. Cells were cultured in a culture flask or culture plate until confluence, and then incubated in serum-free medium for a further 12 hours before the addition of the carvedilol (10 μ M). After incubation with the carvedilol for 1 hour, the cells were stimulated with TNF- α (10 ng/mL).

NF- κ B p65 activation

Cytoplasmic and nuclear protein fractions from HUVEC were separated using an NE-PER kit, as according to the manufacturer's instruction. After preparation, Western blot analysis was performed to detect the protein level of NF- κ B p65 in the cytoplasmic and nuclear fractions.

Immunocytochemistry was performed to confirm NF- κ B activation. HUVEC were pretreated with curcumin for 1 hour, and stimulated with TNF- α for 1 hour. Cells were stained by an indirect immunofluorescence method, fixed for 10 minutes with 2% paraformaldehyde at room temperature and washed three times with PBS. They were then permeabilized for 10 minutes with 0.5% Triton X-100 in PBS, washed three times with PBS and incubated for 10 minutes in 1% BSA in PBS, to block the non-specific binding sites, prior to labeling with the NF- κ B p65 antibody. Primary antibodies were applied for 1 hour at room temperature in 1% BSA-PBS, and the cells then washed three times with PBS, followed by incubation of the Alexa Fluor 568 goat anti-rabbit antibody. The cardiomyocyte nuclei were counterstained with Hoechst 33342. The fluorescence signals were recorded and analyzed using the ImagePro software.

Measurement of intracellular ROS

DCFH-DA is a non-polar compound, which enters the cell and is cleaved to form DCFH. Trapped DCFH is oxidized by oxygen free radicals to produce fluorescent DCF. Cells were cultured on 96-well microplate, preloaded with 10 μ M DCFH-DA, for 30 minutes at 37°C, followed by incubation with TNF- α (10 ng/mL) in the presence (10 μ M) or absence of carvedilol dissolved in DMSO. The fluorescence intensity was analyzed using a fluorescence reader (Fluoroscan Ascent FL, Labsystems, Finland), with excitation and emission at 485 and 538 nm, respectively.

U937 Adhesion assay

U937 cells were labeled with BCECF-AM (10 μ g/mL), for 30 minutes at 37°C, washed and then resuspended in serum-free media. HUVEC were cultured and incubated with reagents on 24-well culture plate, then co-cultured with BCECF-AM-labeled U937 cells for 30 minutes at 37°C. Non-adhering U937 cells were removed, and the 24-well plates washed with PBS. Cells were lysed in 0.1% Triton X-100, in 0.1 M Tris-HCl, at pH 7.4. The fluorescence was measured using a fluorescence reader, with excitation and emission at 510 and 531 nm, respectively.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the HUVEC, using Trizol, as according to the manufacturer's instruction, and then quantified. 1 μ g of total RNA was used as the template for cDNA synthesis at 65°C for 15 minutes, 25°C for 10 minutes, 42°C for 60 minutes and 95°C for 10 minutes, using SuperScript reverse transcriptase, followed by storage of the products at 4°C. The cDNA was amplified by PCR, with specific primers, and the

taining 5% skim milk. The polyvinylidene fluoride was blotted against the indicated antibodies. The protein levels were determined using Western Breeze reagents and an Image Reader (LAS-3000 Imaging System, Fuji Photo Film).

ICAM-1, MCP-1, and IL-8 analysis by enzyme-linked immunosorbent assay (ELISA)

Cells were seeded onto a 96-well plate, pretreated with carvedilol (10 μ M) for 1 hour, followed by the addition of TNF- α . After incubation for 16 h, in CO₂ incubator, the supernatants were collected to measure ICAM-1, MCP-1, and IL-8. The concentrations of cytokines were quantified using a commercially available ELISA development system, according to the manufacturer's protocol.

Results

Carvedilol inhibited NF- κ B p65 nuclear translocation induced by TNF- α

To investigate the effect of carvedilol on the NF- κ B activation, the protein expression of NF- κ B p65 in the cytoplasmic and nuclear fractions were examined. As shown by Western blot and immunocytochemistry, the transcription factor NF- κ B p65 is translocated from the cytosol to the nucleus when activated by TNF- α . NF- κ B p65 activation; however, was inhibited by carvedilol, as well as BAY11-7082, a specific inhibitor of NF- κ B (Fig. 1).

Carvedilol inhibited ROS generation and U937 monocyte adhesion induced by TNF- α

To determine the anti-oxidative effect of carvedilol on TNF- α stimulated HUVEC, the intracellular ROS level was measured. TNF- α increased the intracellular

ROS level at 1 hour ($141 \pm 5.3\%$ vs. control, $p < 0.05$, $n = 4$). Pretreatment with carvedilol for 1 hour significantly reduced the TNF- α induced ROS formation,

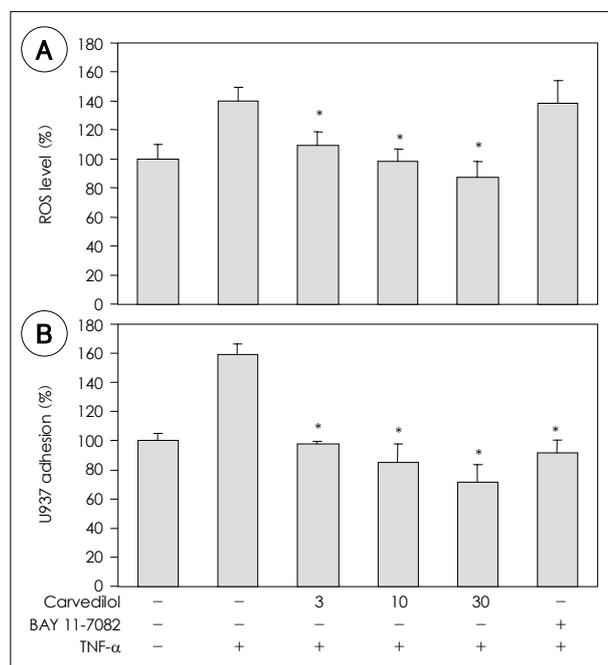


Fig. 2. A: The effect of carvedilol on the intracellular ROS level in TNF- α -activated HUVEC. HUVEC were treated with carvedilol (3, 10, 30 μ M) or BAY 11-7092 (10 μ M) 1 hour prior to the addition of TNF- α (10 ng/mL). The DCFH-DA in the cells was labeled, with fluorescence released due to oxidative reduction. A representative of three experiments is shown. Data are presented as the mean \pm SD ($n = 4$), *: $p < 0.05$ vs. TNF- α alone. B: the effect of carvedilol on the U937 adhesion to TNF- α -activated HUVEC. HUVEC were pretreated with carvedilol, activated with TNF- α (10 ng/mL), and cocultured with BCECF-labeled U937. A representative of three experiments is shown. Data are the means \pm SD ($n = 3$), and represent the quantitative fluorescent results obtained from a microplate reader. *: $p < 0.05$ vs. TNF- α alone. ROS: reactive oxygen species, HUVEC: human umbilical vein endothelial cells, DCFH-DA: dichlorofluorescein diacetate, TNF- α : tumor necrosis factor- α , BCECF: 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein.

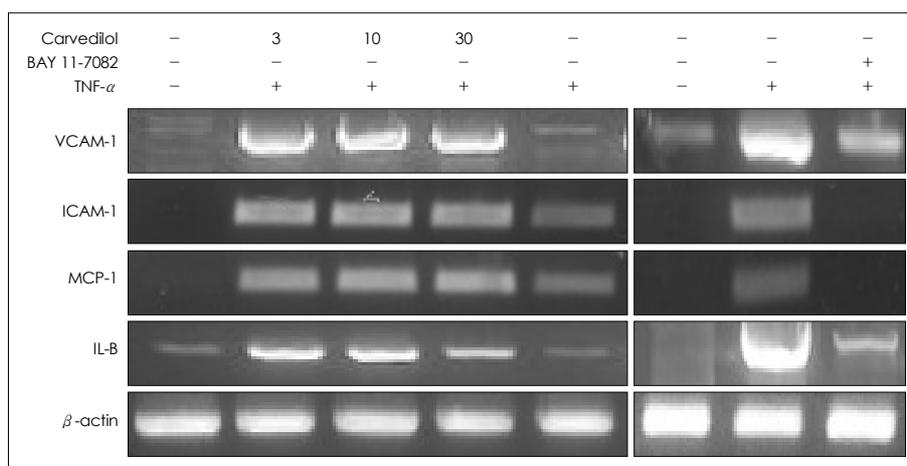


Fig. 3. The effects of carvedilol on the TNF- α induced expressions of VCAM-1, ICAM-1, MCP-1 and IL-8. A, HUVEC were pretreated with carvedilol (3, 10, 30 μ M) or BAY 11-7092 (10 μ M) for 1 hour. Three hours after TNF- α stimulation, total RNA was extracted and used for RT-PCR to determine the mRNA levels (5 separate experiments). TNF- α : tumor necrosis factor- α , VCAM-1: vascular cell adhesion molecule-1, ICAM-1: intercellular adhesion molecule-1, MCP-1: monocyte chemoattractant protein-1, IL-8: interleukin-8, HUVEC: human umbilical vein endothelial cells, RNA: ribonucleic acid, RT-PCR: reverse transcription polymerase chain reaction.

in a dose-dependent manner, and 10 μ M carvedilol almost completely blocked the TNF- α induced ROS formation. Conversely, pretreatment with BAY 11-7082 had no effect on the elevated ROS level caused by TNF- α (Fig. 2A).

To examine the effect of carvedilol on the inflammation induced by TNF- α , an adhesion assay, using the human premonocytic cell line, U937, was carried out. Adhesion of the U937 cells to the HUVEC was enhanced by TNF- α ($161 \pm 7\%$ vs. control, $p < 0.05$, $n = 3$). Pretreatment with carvedilol for 1 hour significantly inhibited the TNF- α induced adhesion, in a

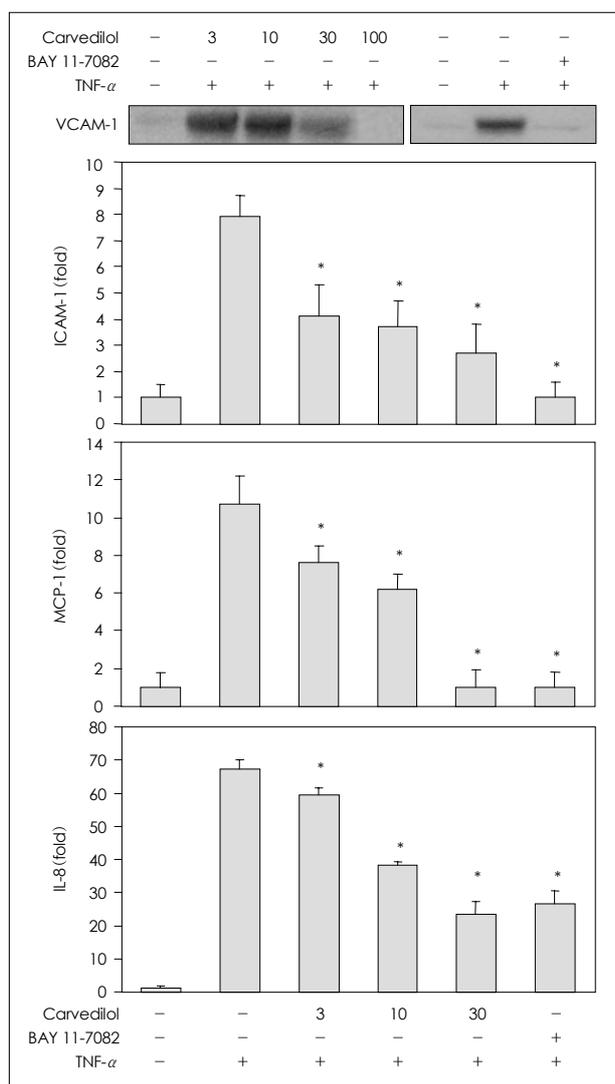


Fig. 4. The whole cell lysates were prepared 16 hours after stimulation, separated on SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with an anti-VCAM-1 antibody. Cells were treated, as indicated, for 16 hours, culture media collected and the ICAM-1, MCP-1 and IL-8 levels measured using an ELISA kit. Data are expressed as the mean \pm SD of triplicate wells. Results are representative of three independent experiments. SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, PVDF: polyvinylidene flouride, VCAM-1: vascular cell adhesion molecule-1, ICAM-1: intercellular adhesion molecule-1, MCP-1: monocyte chemoattractant protein-1, IL-8: interleukin-8.

dose-dependent manner. The BAY 11-7082 also almost completely blocked the U937 adhesion induced by TNF- α (Fig. 2B).

Carvedilol reduced VCAM-1, ICAM-1, MCP-1, and IL-8 expression induced by TNF- α

The expressions of the NF- κ B dependent pro-inflammatory molecules, i.e., VCAM-1, ICAM-1, MCP-1 and IL-8, were determined at the mRNA and protein levels. TNF- α induced both transcriptions and translations of the VCAM-1, ICAM-1, MCP-1 and IL-8. Carvedilol reduced the inductions of the VCAM-1, ICAM-1, MCP-1 and IL-8 mRNAs in the HUVEC treated with TNF- α , in a dose dependent manner (Fig. 3). The BAY 11-7082 also suppressed the transcriptional expressions of VCAM-1, ICAM-1, MCP-1 and IL-8.

The expressions of the VCAM-1, ICAM-1, MCP-1 and IL-8 proteins were markedly increased in the TNF- α stimulated cells. The TNF- α stimulated cells treated with 10 μ M carvedilol demonstrated full inhibition of the VCAM-1 protein, as well as dose-dependent inhibition of the ICAM-1, MCP-1 and IL-8 (Fig. 4). TNF- α -activated expressions of the VCAM-1, ICAM-1, MCP-1 and IL-8 proteins were inhibited when the HUVEC were pretreated with BAY 11-7082.

Carvedilol inhibited JNK activation and Bcl-2 reduction induced by TNF- α

The stress-activated signaling molecules, such as JNK and Bcl-2, were examined to see if they were modulated by carvedilol. TNF- α increased the JNK phosphorylation within 15 minutes, but reduced the Bcl-2 expression within 24 hours. Carvedilol inhibited the

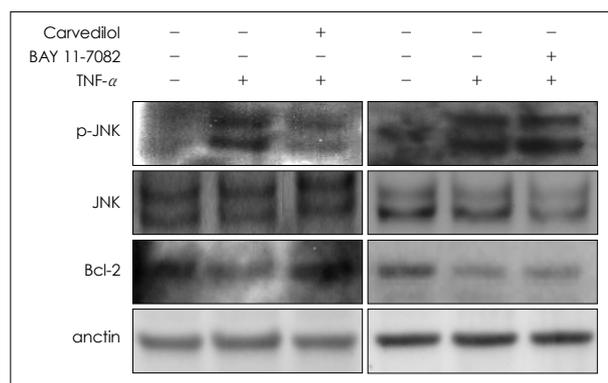


Fig. 5. The effects of carvedilol on JNK phosphorylation and Bcl-2. HUVEC were pretreated with carvedilol (10 μ M) or BAY 11-7082 (10 μ M) and then cultured with TNF- α , as indicated, for 15 minutes or 24 hours, to detect JNK phosphorylation and the Bcl-2 level, respectively. The whole cell lysates were prepared, separated on SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with an anti-phosphorylated JNK or Bcl-2 antibody. Results are representative of three independent experiments. JNK: c-Jun N-terminal protein kinase, HUVEC: human umbilical vein endothelial cells, TNF- α : tumor necrosis factor- α , SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, PVDF: polyvinylidene flouride.

TNF- α induced JNK phosphorylation and Bcl-2 reduction, while with BAY 11-7082 the phosphorylation of JNK and Bcl-2 protein expression patterns remained unchanged (Fig. 5).

Discussion

Inflammatory reactions, such as the expressions of cell adhesion molecules and cytokines with monocyte adhesion, have been observed in atherosclerotic lesions. Increased pro-inflammatory cytokines and the expressions of adhesion molecules, such as VCAM and ICAM, on endothelial cells participate in atherogenesis.¹¹⁾¹²⁾ In this study, VCAM-1, ICAM-1, MCP-1 and IL-8, which have been reported to be regulated in a strictly NF- κ B dependent fashion, were chosen as parameters of inflammation.¹³⁾

Many studies have revealed carvedilol to be an anti-oxidative, the cardioprotective effects of which are mediated by the inhibition of NF- κ B activation.¹⁴⁾¹⁵⁾ ROS are thought to be involved in the development of a number of pathological conditions and diseases, especially of the cardiovascular system.¹⁶⁾ The administration of anti-oxidative drugs to treat or prevent such conditions is of great interest. Singh et al.¹⁷⁾ reported that carvedilol significantly attenuated lipid peroxidation and protected against severe depletion of the antioxidant enzyme pool, such as reduced glutathione, glutathione reductase, catalase and SOD in glycerol-treated rats.

NF- κ B has been suggested as the major transcriptional factor in chronic inflammatory diseases related to endothelial adhesion molecules, such as E-selectin, ICAM-1 and VCAM-1, as well as of chemokines, such as monocyte-chemoattractant protein-1 (MCP-1) and IL-8.³⁾ In this study, carvedilol blocked the nuclear translocation of NF- κ B p65 stimulated by TNF- α , which in turn reduced the expressions of VCAM-1, ICAM-1, MCP-1, and IL-8 at the transcriptional level. Carvedilol was also found to attenuate the increase in the intracellular ROS level, in a dose-dependent manner, in TNF- α treated HUVEC.

Carvedilol may inhibit early events in the atherosclerotic process, possibly due to its interference with NF- κ B-dependent transcription. The effects of BAY 11-7082 on the action of TNF- α were tested and compare with those of carvedilol. BAY 11-7082, an irreversible inhibitor of I κ B α phosphorylation, also inhibited the NF- κ B p65 translocation induced by TNF- α . BAY 11-7082 inhibited the U937 adhesion, and the expressions of VCAM-1, ICAM-1, MCP-1 and IL-8; however, unlike carvedilol, the elevated ROS level induced by TNF- α was unchanged.

JNK, a member of MAPKs, plays important roles in signaling pathways for apoptosis, transformation, de-

velopment, immune activation, inflammation and adaptation to environmental changes.⁵⁾ This enzyme is important in the pathways regulating cell death, which are usually activated by cytokines, such as TNF- α and IL-1, and genotoxic stresses, such as UV. In this report, JNK phosphorylation due to TNF- α increased within 15 minutes, but decreased to basal levels within 1 hour (data not shown). Pretreatment of carvedilol for 1 hour significantly inhibited JNK phosphorylation. In addition, the level of Bcl-2, one of the regulatory proteins related to cell survival, was also examined.¹⁸⁾ TNF- α has been reported to specifically suppress the expression of Bcl-2 and induce a rise in the concentration of cytoplasmic Ca²⁺ due to the release of Ca²⁺ from intracellular Ca²⁺ stores in hepatoma cells.¹⁹⁾ Carvedilol rescued the Bcl-2 protein, which was significantly down-regulated by TNF- α . BAY11-7082; however, did not alter the ROS production, JNK phosphorylation or Bcl-2 down-regulation in TNF- α stimulated HUVEC.

Although carvedilol has additional anti-oxidative activity, which is independent of its anti-hypertensive effects, it is not clear whether ROS might enhance the action of TNF- α . To address this question, the effect of antioxidants on TNF- α -induced events should be examined.

In our data, carvedilol almost completely inhibited the U937 monocyte adhesion to TNF- α -activated endothelial cells, at least in part, by the inhibition of the expressions of VCAM-1 and ICAM-1. Accordingly, the Western blot data for VCAM-1 supported the in vitro adhesion results. This study has provided insights to the role exerted by carvedilol, and the beneficial effects on TNF- α -stimulated HUVEC through its antioxidant activity, NF- κ B inhibition and JNK inhibition, as well as α -adrenoreceptor blockade. From the present data, it is suggested that carvedilol could significantly reduce the expressions of inflammatory molecules induced by TNF- α . Therefore, the effects of carvedilol might be suggested to be related with its antioxidant activity, contributing significantly to its clinical benefit. Carvedilol blocked the expression of cytokines by direct modulation at the gene transcription level.

In conclusion, carvedilol has efficacy in inhibiting TNF- α -induced inflammatory responses, as well as apoptosis. These observations suggest that carvedilol plays a significant role in the attenuation of inflammatory disease and apoptosis in the cardiovascular system.

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