

KR-31543 reduces the production of proinflammatory molecules in human endothelial cells and monocytes and attenuates atherosclerosis in mouse model

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Abbreviations: HUVEC, human umbilical vein endothelial cell; MCP-1, monocyte chemoattractant protein-1; ROS, reactive oxygen species

Abstract

KR-31543, (2S, 3R, 4S)-6-amino-4-[N-(4-chlorophenyl)-N-(2-methyl-2H-tetrazol-5-ylmethyl) amino]-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2H-1-benzopyran is a new neuroprotective agent for ischemia-reperfusion damage. It has also been reported that KR-31543 has protective effects on lipid peroxidation and H₂O₂-induced reactive oxygen species production. In this study, we investigated the anti-inflammatory and anti-atherogenic properties of KR-31543. We observed that KR-31543 treatment reduced the production of MCP-1, IL-8, and VCAM-1 in HUVECs, and of MCP-1 and IL-6 in THP-1 human monocytes. We

also examined the effect of KR-31543 on monocytes migration *in vitro*. KR-31543 treatment effectively reduced the migration of THP-1 human monocytes to the HUVEC monolayer in a dose-dependent manner. We next examined the effects of this compound on atherogenesis in LDL receptor deficient (*Ldlr*^{-/-}) mice. After 10 weeks of western diet, the formation of atherosclerotic lesion in aorta was reduced in the KR-31543-treated group compared to the control group. The accumulation of macrophages in lesion was also reduced in KR-31543 treated group. However, the plasma levels of total cholesterol, HDL, LDL, and triglyceride were not affected by KR-31543 treatment. Taken together, these results show that KR-31543 has anti-inflammatory properties on human monocytes and endothelial cells, and inhibits fatty streak lesion formation in mouse model of atherosclerosis, suggesting the potential of KR-31543 for the treatment for atherosclerosis.

Keywords: atherosclerosis; endothelial cells; KR-31543; monocytes; transendothelial and transepithelial migration

Introduction

Atherosclerosis is a representative chronic vascular inflammatory disease. The early stages of the disease involve the recruitment of monocytes and lymphocytes into the arterial intima, and localized accumulation of the cells (Ross, 1999). This early inflammatory step of atherosclerosis is mostly initiated by reactive oxygen species (ROS)-induced oxidative modification of lipoproteins in subintimal space (Hansson and Hermansson, 2011). Oxidized low-density lipoprotein (LDL) particularly induces the expression of inflammatory molecules, including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, interleukin-8 (IL-8), macrophage chemoattractant protein-1 (MCP-1), etc. (Terkeltaub *et al.*, 1994; Khan *et al.*, 1995; Claise *et al.*, 1996; Wang *et al.*, 1997). These multiple processes influence the recruitment of monocytes to the intima of arterial wall.

Therefore, the blockage of oxidative modification of LDL is an important strategy for reducing early stage of atherosclerosis.

(2S, 3R, 4S)-6-amino-4-[4-chloro-(2-methyltetrazol-5-ylmethyl)phenyl]amino]-3,4-dihydro-3-hydroxy-2-dimethoxy-methyl-2H-1-benzopyran, or KR-31543, synthesized by the Korea Research Institute of Chemical Technology, was originally developed as a neuroprotective agent for ischemia and reperfusion damage. Possessing both antioxidant and potassium channel-modulating properties, KR-31543 protected cultured rat cortex neurons against iron-induced oxidative injury *in vitro*, and significantly reduced infarct volume following occlusion in the rat model of transient cerebral ischemia (Kim *et al.*, 2002; Lee *et al.*, 2003). Since the inhibition of ROS generation can attenuate the generation of atherosclerotic lesions (Park and Oh, 2011), the anti-oxidative activity of KR-31543 led us to investigate its anti-atherogenic activity. Here we first demonstrated the anti-inflammatory and anti-atherosclerotic potential of KR-31543 using THP-1 human monocytes, HUVECs, and LDL receptor deficient (*Ldlr*^{-/-}) mice.

Results

KR-31543 down-regulated the production of proinflammatory molecules in HUVECs and THP-1 monocytes

In a previous paper, KR-31378, an antioxidant and potassium channel modulator similar to KR-31543, has been reported to reduce the production of inflammatory molecules in cytokine-stimulated leukocytes and endothelial cells directly (Kim *et al.*, 2004). In this study, the expression levels of two chemotactic molecules, MCP-1 and IL-8, were determined in HUVECs treated and untreated with several indicated concentrations of KR-31543, having no cytotoxicity in mitochondrial tetrazolium assay. Incubation of HUVECs with KR-31543 for 2 h prior to TNF- α stimulation resulted in a significant decrease in the secretion of MCP-1 in a dose-dependent manner (Figure 1B). Moreover, KR-31543 markedly decreased the production of IL-8 in HUVECs (Figure 1C). VCAM-1 expression was also decreased at high concentration of KR-31543, but there was no change in lower concentration (Figure 1D). It was also tested whether KR-31543 decreased cytokine production in macrophages, and found that KR-31543 effectively reduced MCP-1 and IL-6 productions in THP-1 human monocyte (Figures 1E and 1F).

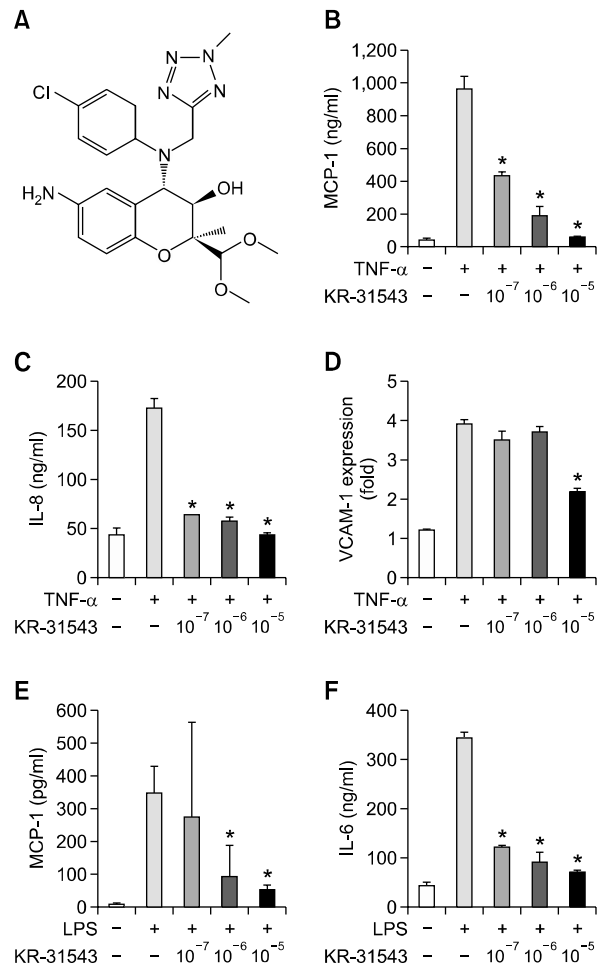


Figure 1. Effect of KR-31543 on the expressions of proinflammatory molecules in human endothelial cells and THP-1 monocytes. (A) The chemical structure of KR-31543. (B-D) The effect of KR-31543 treatment on several proinflammatory molecules including (B) MCP-1 and (C) IL-8 and (D) VCAM-1 in HUVECs. (E, F) The effect of KR-31543 treatment on the expression of (E) MCP-1 and (F) IL-6 in THP-1 human monocytes. *indicates $P < 0.01$ compared to control group.

KR-31543 reduced migration of macrophage induced by TNF- α

In this study, *in vitro* leukocyte migration and adhesion assays were performed to see the effects of KR-31543 on early inflammatory process. First, it was investigated whether KR-31543 reduces the adhesion of monocytes to endothelial cells. We found that although 10⁻⁶ M of KR-31543 did not affect the expression of VCAM-1 (Figure 1D), KR-31543 effectively reduced the attachment of THP-1 human monocytes to HUVEC monolayers at this concentration (Figure 2A). It seems that attenuated expressions of IL-8 and MCP-1 by 10⁻⁶ M of KR-31543 are responsible for reduced monocyte attachment. The observation that KR-31543 reduced THP-1 human monocyte adhesion to HUVEC mon-

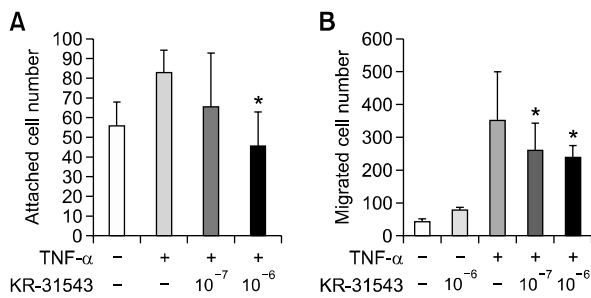


Figure 2. The effect of KR-31543 on monocyte adhesion and transmigration. (A) Monocyte adhesion assay. HUVECs were pre-incubated with KR-31543 (10^{-6} and 10^{-7} M) for 2 h, and then stimulated with TNF- α for 12 h. Next THP-1 human monocytes were added to the culture. After 20 min, unbound cells were washed and the remaining attached cells were counted in five randomly selected optical fields per well. (B) Monocyte migration assay. THP-1 human monocytes were loaded into the upper chamber of Transwell plates and the lower chambers were filled with conditioned medium from HUVECs treated with KR-31543 (10^{-6} or 10^{-7} M) or left untreated for 2 h and subsequently activated with TNF- α for 12 h. *indicates $P < 0.05$ compared to TNF- α stimulated cells without KR-31543 treatment.

layers prompted an examination of whether KR-31543 could also affect cell migration. HUVECs were pretreated with KR-31543 for 2 h, then stimulated with TNF- α for 12 h; stimulated with TNF- α alone; or left untreated. The conditioned culture media were then placed in the bottom chambers of a ChemoTx plate. THP-1 human monocytes were loaded onto the 5- μ m pore filter and incubated for 6 h. Incubation with TNF- α -conditioned medium resulted in approximately 6-fold increase of migrated THP-1 human monocytes compared to the untreated HUVEC conditioned medium. In contrast, a conditioned medium taken from cells pre-incubated with KR-31543 markedly reduced the migration of THP-1 human monocytes in a dose-dependent manner (Figure 2B).

KR-31543 significantly reduced the atherosclerotic lesion and lesional macrophage accumulation in atherogenic diet-fed *Ldlr*^{-/-} mice

To analyze the pharmacokinetics of KR-31543, the mice were administered 3 mg of KR-31543 once orally. This dose was equivalent to the daily amount ingested from the diet used in mouse study (KR-31543 1% w/w). The plasma KR-31543 level in the treated animals was 1.0×10^{-7} M at 24 h after giving the chemical. Therefore, considering that KR-31543 was effective to reduce inflammatory cytokines from endothelial cells and macrophages at the concentration of 1×10^{-7} M, it seems that the administered KR-31543 effectively attenuate the vascular inflammatory processes. To determine the effect of KR-31543 on the development of atherosclerosis, atherosclerotic lesions in aortic

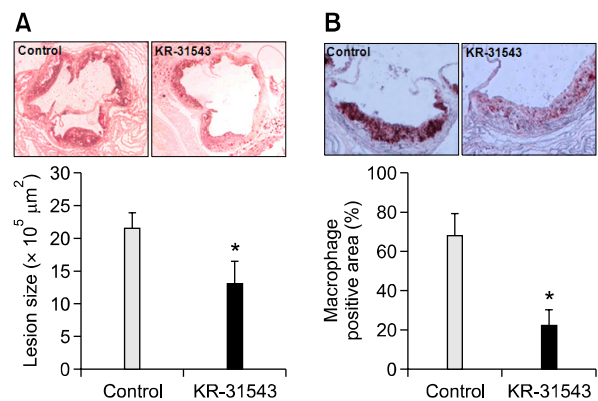


Figure 3. The anti-atherogenic potential of KR-31543 in *Ldlr*^{-/-} mice. (A) Representative figures of Oil red O-positive atherosclerotic lesion from Control ($n = 8$) and KR-31543 groups ($n = 8$). The mean lesion size was significantly reduced in KR-31543 group compared to control group. (B) Representative immunohistochemical staining of macrophages in control ($n = 8$) and KR-31543-treated ($n = 8$) mice are shown. KR-31543 effectively reduced the macrophage accumulation in the lesion. The graph shows the percent mean area of macrophage accumulation in total atherosclerotic lesion of each mouse. *indicates $P < 0.05$ compared to control group.

vessels from the control and KR-31543 groups which had been fed a Western diet for 10 weeks were analyzed. Necropsy and histopathological examinations did not reveal notable lesions in the parenchymal organs of both groups of mice (data not shown). In addition, daily food intake and weight gain was similar between the two groups (data not shown). Measurement of the lesion size by computer-associated morphometry revealed that the mean lesion area of the aortic sinus in the KR-31543 group was smaller than that of the control group by around 40% (Figure 3A). The results from cell study prompted a measurement of the macrophage accumulation in atherosclerotic lesion. The lesions were immunostained with anti-macrophage antibodies (MOMA-2) and measured the macrophage positive area in the aortic sinus to determine whether KR-31543 treatment reduced monocyte recruitment to the lesion. The macrophage positive area was reduced significantly in the KR-31543 group compared to the control group (Figure 3B).

KR-31543 did not affect plasma lipid levels

Since lipid lowering agent also can reduce the formation of atherosclerotic lesion, the plasma lipid profiles were analyzed in KR-31543 treated mice. KR-31543 supplementation did not affect plasma concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride (Figure 4). These results suggest that KR-31543 reduces atherosclerosis not by modulating plasma lipid profiles, but by its

anti-inflammatory properties.

Taken together, our results suggest that KR-31543 reduces the atherosclerotic lesion formation possibly by disturbing the inflammatory processes leading to attenuation of atherosclerosis (Figure 5).

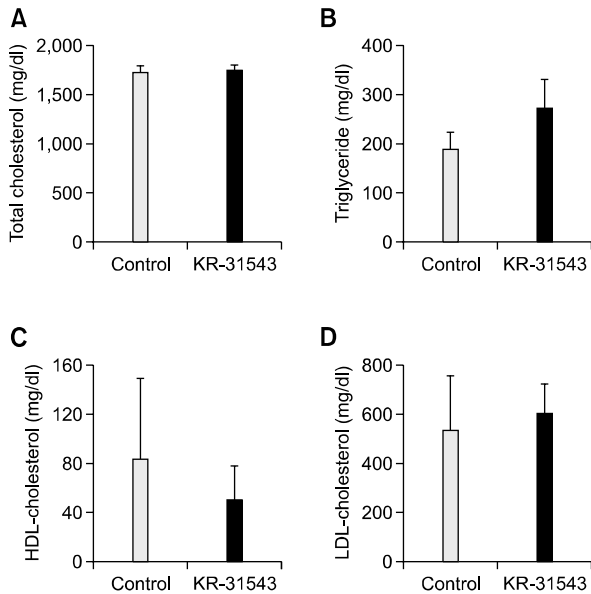


Figure 4. The effect of KR-31543 on plasma lipid profiles. (A) Total cholesterol, (B) Triglyceride, (C) HDL-cholesterol, and (D) LDL-cholesterol were measured by blood chemical analyzer. There was no significant difference between groups.

Discussion

Many previous studies have suggested that antioxidants inhibit inflammatory responses by blocking the expression of pro-inflammatory molecules, leading to attenuation of the atherosclerotic lesion formation (Sugamura and Keane, 2011). In this study, we demonstrated that the antioxidant KR-31543 decreases diet-induced lesion formation and macrophage infiltration in *Ldlr*^{-/-} mice. Treatment with KR-31543 decreases monocyte migration and attachment to endothelial cells *in vitro*, which may be attributed to a reduction in the production of IL-8, IL-6 and MCP-1.

Hyperlipidemia is one of the major risk factors for atherosclerosis, and many studies have suggested that lipid-lowering drugs are effective to reduce atherosclerosis (Ross and Harker, 1976; Gotto and LaRosa, 2005; Hackam, 2006; Ford *et al.*, 2007). However, our analysis of the plasma level of total cholesterol, HDL, LDL, and triglycerides revealed that treatment with KR-31543 did not influence the lipid profiles of the mice fed high fat diet. Thus, it was concluded that unlike the lipid profile-modulating drugs like statins, KR-31543 exerts its anti-atherogenic effect through mechanisms other than improvement of lipid profiles.

In this study, our cell adhesion assay demonstrated that KR-31543 efficiently reduced THP-1 monocyte attachment to endothelial cells stimulated by TNF- α without attendant cytotoxicity. These results led us to investigate the effect of KR-31543 on the transmigration of monocytic cells. In our migration

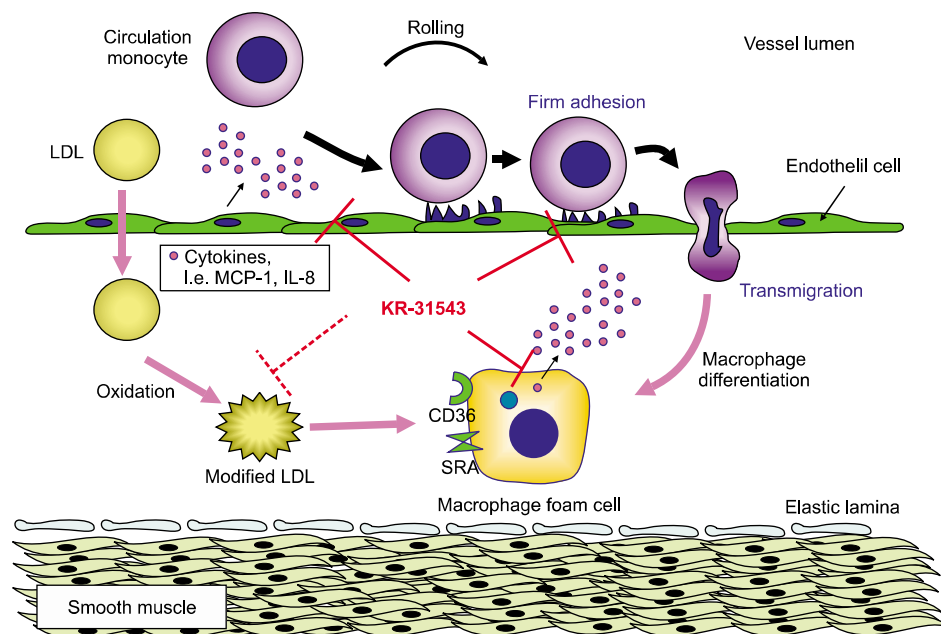


Figure 5. Suggested possible mechanism for the anti-atherogenic activity of KR-31543. KR-31543 attenuates the atherosclerotic lesion formation by reducing the production of cytokines and disturbing the adhesion and transmigration of monocytes (red line). The anti-oxidative activity of KR-31543 may also reduce the oxidation of LDL, which can be an additional possible mechanism of its antiatherogenic characteristic (dashed red line).

assays, we found that the conditioned media from HUVEC cells challenged with TNF- α significantly increased THP-1 migration, whereas pretreatment with KR-31543 decreased the number of migrating cells in a dose-dependent manner. Taken together, these results suggest that KR-31543 may block the monocyte adhesion and transmigration induced by the factors derived from endothelial cells and monocytes. Consistent with this data, KR-31543 effectively reduced TNF- α -induced production of chemo-attracting molecules in HUVECs. The secretion of IL-8, a well-known regulator of neutrophil migration and a mediator of acute inflammatory reactions, was significantly reduced. Furthermore, the production of MCP-1, a mediator in chronic inflammation was decreased by pre-incubation with KR-31543 not only in HUVECs, but also in THP-1 human monocytes (Scapini *et al.*, 2000; Ito and Ikeda, 2003). Therefore, we speculate that KR-31543 acts as a regulator of both endothelial cell and monocyte activity by down-regulation of monocyte chemo-attractant production, resulting in a synergistic suppression of recruitment *in vivo*. This hypothesis is supported by the observation that in the lesions formed in *Ldlr*^{-/-} mice, macrophage accumulation was reduced in the KR31543-treated group as compared to the control group.

In this study, it is not clarified how KR-31543 exerts an inhibitory effect on monocyte adhesion on endothelial cell monolayer and transmigration. Previously, it has been well-known that hydrogen peroxide and oxygen radicals are produced during inflammatory processes and play second messengers directly or indirectly mediating the activation of pro-inflammatory nuclear transcription factors, such as NF- κ B (Schreck *et al.*, 1991). Therefore, the reduction of reactive oxygen species (ROS) by anti-oxidants, such as flavonoid, α -tocopherol, ascorbate, and gallate, has potential anti-atherogenic effects *via* decreased pro-inflammatory molecules needed for monocyte migration and adhesion (Gerritsen *et al.*, 1995; Murase *et al.*, 1999; Carr *et al.*, 2000). MCP-1 and IL-8, which play roles in the recruitment of monocytes, are regulated *via* the TNF- α -mediated NF- κ B signaling pathway (Collins and Cybulsky, 2001; Wajant *et al.*, 2003). Thus, we speculate that the mechanism of the anti-atherogenic activity of KR-31543 involves antioxidant action leading to a reduction in the activation of pro-inflammatory transcriptional factors such as NF- κ B.

In conclusion, we showed that KR-31543 effectively reduced atherosclerotic lesion formation and macrophage accumulation in the lesions *via* its anti-inflammatory properties, which modulate multiple cellular activities and interactions. Our results indicate that in addition to its neuro-protective

characteristics, KR-31543 may be a valid therapeutic candidate for the treatment of atherosclerosis.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (BioWhittaker, Walkersville, MD) and maintained in EGM-2 Bullet kit media (BioWhittaker) at 37°C with 5% CO₂. All HUVECs used in this study were between passages 3 and 7.

THP-1 human monocytes were obtained from the American Type Culture Collection. THP-1 human monocytes were cultured in RPMI1640 containing 10% Fetal Bovine Serum supplemented with 100 units/ml of penicillin and 100 μ g/ml of streptomycin (Gibco BRL, CA).

Chemicals

KR-31543 (Figure 1A, generously donated by the Korea Research Institute of Chemical Technology, Daejeon, Korea) was dissolved in dimethyl sulfoxide as a 100 mM stock solution and kept at -20°C.

Animal model

Ldlr^{-/-} mice were randomly divided into two groups (control $n = 8$, KR-31543 group $n = 8$). The control group was fed a Western diet (CRF-1 supplemented with 0.15% cholesterol and 20% fat, Oriental Yeast Co. Ltd., Tokyo, Japan), while the KR-31543 group was fed the same diet supplemented with 0.1% (w/w) KR-31543. All mice were given water and food ad libitum. After 10 weeks, the mice were killed, and plasma chemical profiles were generated using an automatic blood chemical analyzer (HITACHI, Japan). Parenchymal organs were also harvested for histopathological examination. This study was performed in accordance with the recommendations from the Declaration of Helsinki. All animal study protocols were approved by the Institutional Animal Care and Usage Committee of Ewha Womans University (Seoul, Korea).

Assessment of atherosclerosis

Fatty streak lesions were quantified by evaluating the lesion size in the aortic sinus, as previously described (Choi *et al.*, 2003). The heart and aorta were perfused with phosphate-buffered saline (PBS) for 10 min, with 4% paraformaldehyde for 5 min, and then promptly removed. After fixation for 1 day in 10% buffered neutral formalin, the hearts and aortas were embedded in OCT medium (Life Science International, UK) and frozen at -70°C. All samples were sectioned using a cryostat at 20°C, and six consecutive 9 μ m-thick sections were cut from the aorta where the valve cusp becomes visible. Atherosclerotic plaques were stained with Oil red O and counter-stained with Harris hematoxylin. The lesion area was then quantified by computer-assisted morphometry (Image pro plus, MD) and the average lesion size was calculated for each animal.

Measurement of macrophage accumulation in atherosclerotic lesions

Frozen sections were stained with a rat monoclonal antibody against mouse macrophages (MOMA-2, Serotec Inc., NC) using the ABC method (Novastatin Super ABC kit, Novocastra Lab. Ltd., UK). The macrophage contents of lesions was calculated as the percentage of mean MOMA-2 positive area versus mean total lesion area using five consecutive sections per mouse by computer-aided morphometric analysis.

Macrophage adhesion assay

Adhesion of THP-1 human monocytes to HUVEC monolayer was assayed as described previously (Che *et al.*, 2002; Kim *et al.*, 2004). HUVECs were plated on 6-well plates at a density of 1×10^5 cells per well and cultured to 90% confluence in EGM-2 Bullet kit media. After 2 h of KR-31543 treatment, the cells were incubated with 20 ng/ml of TNF- α for another 12 h. THP-1 human monocytes in the exponential growth phase were washed with serum-free DMEM and then re-suspended in the same medium to 2×10^4 cells/ml. The THP-1 human monocyte suspension (1 ml) was added into the wells containing HUVEC monolayers and incubated for 20 min at 37°C. Unbound cells were washed three times with serum-free DMEM. The total number of adherent cells was counted in five randomly selected optical fields per well.

Monocyte migration assay

Monocyte migration assay was performed as described previously (Kim *et al.*, 2004). Briefly, the migration of THP-1 human monocytes was assayed using the ChemoTx kit (Neuro Probe, Inc. MD) in a 96-well plate housing a collagen-precoated polycarbonate filter with 5.0- μ m pores, according to the manufacturer's instructions. The lower chambers were filled with 30 μ l of medium obtained from cultured human umbilical vein endothelial cells (HUVECs), treated with KR-31543 for 2 h, and subsequently stimulated with TNF- α (Promega). The filter membranes were loaded with 1.25×10^4 cells in 25 μ l. After incubation for 6 h at 37°C, cells that had migrated into the lower chamber were counted by flow cytometry.

Measurement of proinflammatory molecules

THP-1 human monocytes were differentiated into macrophages by PMA treatment for 24 h. Then LPS and indicated doses of KR-31543 were added to culture media. After pre-incubation with the indicated dose of KR-31543, HUVECs were stimulated by 3 ng/ml of TNF- α . The levels of IL-8 and MCP-1 were determined by ELISA method (R&D) according to the manufacturer's protocol. The measurement of VCAM-1 expression was performed as described previously (Kim *et al.*, 2004). Briefly, HUVECs were treated with KR-31543 for 2 h and then stimulated with 1 ng/ml of TNF- α for 12 h. The cells were collected and stained for 1 h on ice with mouse anti-human VCAM-1 monoclonal antibody (PharMingen) or mouse IgG1 κ (PharMingen) as an isotype control. After this, cells were

washed and then analyzed by FACScaliber (BD).

Statistical analysis

Comparisons between two groups were made using a two-tailed Student's *t* test. ANOVA followed by the Fisher's Protected Least Significant Difference method was used for multiple comparisons. All data in this study are expressed as the mean \pm standard deviation. Values of *P* < 0.05 were considered significant.

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