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NADPH Oxidase and Mitochondrial ROS are Involved in the TNF-α-induced Vascular Cell Adhesion Molecule-1 and Monocyte Adhesion in Cultured Endothelial Cells

Jae Hyeon Yu[1]*, Cuk Seong Kim[2]*, Dae Goon Yoo[2], Yun Jeong Song[2], Hee Kyoung Joo[2], Gun Kang[2], Ji Yoon Jo[2], Jin Bong Park[2], and Byeong Hwa Jeon[2]

Departments of [1]Thoracic Surgery, [2]Physiology, College of Medicine, Chungnam National University, Daejeon 301-131, Korea

Atherosclerosis is considered as a chronic inflammatory process. However, the nature of the oxidant signaling that regulates monocyte adhesion and its underlying mechanism is poorly understood. We investigated the role of reactive oxygen species on the vascular cell adhesion molecule-1 (VCAM-1) and monocyte adhesion in the cultured endothelial cells. TNF-α at a range of 1~30 ng/ml induced VCAM-1 expression dose-dependently. BCECF-AM-labeled U937 cells firmly adhered on the surface of endothelial cells when the endothelial cells were incubated with TNF-α (15 ng/ml). Ten μmol/L of SB203580, an inhibitor of p38 MAPK, significantly reduced TNF-α-induced VCAM-1 expression, compared to the JNK inhibitor (40 μmol/L of SP60015) or ERK inhibitor (40 μmmol/L of U0126). Also, SB203580 significantly inhibited TNF-α-induced monocyte adhesion in HUVEC. Superoxide production was minimal in the basal condition, however, treatment of TNF-α induced superoxide production in the dihydroethidine-loaded endothelial cells. Diphenyleneiodonium (DPI, 10 μmol/L), an inhibitor of NADPH oxidase, and rotenone (1 μmol/L), an inhibitor of mitochondrial complex I inhibited TNF-α-induced superoxide production, VCAM-1 expression and monocyte adhesion in the endothelial cells. Taken together, our data suggest that NADPH oxidase and mitochondrial ROS were involved in TNF-α-induced VCAM-1 and monocyte adhesion in the endothelial cells.

Key Words: Reactive oxygen species, p38 MAPK, Endothelial cells, Vascular cell adhesion molecule-1, Tumor necrosis factor-α

INTRODUCTION

Atherosclerosis is considered as a chronic inflammatory process (Ross, 1999), and the endothelium plays an important role in its initiation (Gimbrone, 1995). Various atherogenic stimuli, such as modified low density lipoproteins, oxidative free radicals, homocysteine, and infectious agents, lead to the development of endothelial dysfunction (Ross, 1999). Many of these stimuli cause endothelial cell activation, which is defined as functional and antigenic changes in endothelial cells, promoting monocyte adhesion. Endothelial activation involves the coordinated induction of genes encoding for leukocyte adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and chemotactic factors such as monocyte chemoattractant protein-1.

Reactive oxygen species (ROS) such as the hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), superoxide ($O_2^{\cdot-}$) and H_2O_2, play a major role in vascular biology because of their effects on signal transduction, gene expression, cell growth, and apoptosis, as well as endothelium-dependent vasodilation in vascular cells. ROS may serve as a common intracellular messenger for various redox-sensitive transcription pathways that lead to adhesion molecule expression in vascular endothelial cells (Ross, 1999; Harrison et al, 2003). Accumulating *in vitro* and *in vivo* evidence showed that substances with antioxidant activity such as probucol, flavonoid, carvedilol and ginkgo biloba extract could scavenge intracellular ROS and inhibit endothelial adhesiveness to monocytes by reducing the expression of various adhesion molecules (Chen et al, 2003; Chen et al, 2004). In mammalian cells, MAPKs are strongly activated by growth factors, environmental stresses and inflammatory cytokines (Cybulsky et al, 2001). Among the cytokines, lipopolysaccharide or tumor necrosis factor-α (TNF-α) can activate MAPKs in the signaling pathway, leading to expression of several cytokines (Baud & Karin, 2001; Kim et al, 2006b), and also can induce monocyte adhesion in

Corresponding to: Byeong Hwa Jeon, Department of Physiology, College of Medicine, Chungnam National University, 6, Munhwa-dong, Jung-gu, Daejeon 301-131, Korea. (Tel) 82-42-580-8214, (Fax) +82-42-585-8440, (E-mail) bhjeon@cnu.ac.kr
*Equally contributed in this paper.

ABBREVIATIONS: HUVEC, human umbilical vein endothelial cells; MAPK, mitogen activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; VCAM- 1, vascular cell adhesion molecule-1.

the endothelial cells (Kim et al, 2006a). However, the oxidant signaling that regulates monocyte adhesion in the endothelial cells is poorly understood. Therefore, we investigated the role of ROS and MAPKs in the monocyte adhesion in the cultured human endothelial cells.

METHODS

Cells culture

Human umbilical venous endothelial cells (HUVECs) were grown in EBM-2 supplemented with 2% FBS and EGM-2 Singlequots (Cambrex Bioscience, USA). All cultured cells were maintained at 37°C in 5% CO_2 incubation. U937 monocyte cell line was grown in Dulbecco's minimum essential medium (DMEM) with 10% heat-inactivated FBS, 100 U/ml ampicillin, and 100 mg/ml streptomycin (Jeon et al, 2004; Kim et al, 2006a; Kim et al, 2006b).

Western blot analysis

Equivalent amounts of total protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gel were transferred to nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Richmond, CA, USA). After blocking of nonspecific binding, nitrocellulose blots were incubated with a primary monoclonal antibody. And the blot was incubated with a secondary polyclonal anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (Amersham International, Germany). Bound immunocomplexes were visualized by ECL reagents (Amersham Pharmacia Biotech, USA). Anti-β-actin was used as an internal control to monitor equal protein sample loading.

Detection of superoxide production

Intracellular superoxide was detected by using the superoxide-sensitive fluorophore dihydroethidine (DHE) as a probe (Kim et al, 2006a). For detection of DHE fluorescence, cells were grown in chamber slides (2×10^5 cells/well) (Nalge Nunc International). Cells were rinsed three times with 3 ml of Krebs-HEPES buffer and then exposed to 10 μM DHE for 20 minutes at 37°C in Krebs-HEPES buffer. DHE was then washed from the cells to avoid absorption of any extracellular oxyethidium formed by autooxidation of DHE, and the cells were imaged using a confocal microscope.

Monocyte adhesion assay

U937 cells were fluorescently labeled with 2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) for the quantitative adhesion assay (Kim et al, 2006a; Kim et al, 2006b). Thus the cells (10^7 cells/ml) were incubated with 1 μM BCECF-AM in DMEM medium for 30 minutes at 37°C in 5% CO_2. Then they were washed 3 times with phosphate buffered saline to remove excess dye. Finally, the cells were resuspended in DMEM medium at a density of 10^6 cells/ml. HUVEC were cultured in 24-well plate until confluent. After incubation, TNF-α (15 ng/ml) was added to the wells and incubated for another 18 hours. Then HUVECs were co-incubated with BCECF/AM-labeled U937 cells for 30 minutes at 37°C. Non-adhering U937 cells were removed, and the 24-well plates were washed twice with DMEM. The cells were visualized by confocal microscope. The adherent cells were determined by measuring the fluorescent signals.

Drugs

VCAM-1 and β-actin antibody were obtained from Santa Cruz (California, DA. USA). SB203580, U0126, and SP-600125 were purchased from Calbiochem (Ra Jolla, CA, USA). Dihydroethidine, diphenyleneiodonium (DPI), allopurinol and rotenone were purchased from Sigma (St. Louis, MO. USA). 2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes (USA).

Statistical analysis

Data is presented as mean$\pm$SE for the indicated number of independently performed experiments. Differences were considered statistically significant if $p<0.05$, using analysis of Student's *t*-test.

RESULTS

TNF-α induced VCAM-1 and monocyte adhesion in HUVECs.

To explore whether TNF-α induces the expression of VCAM-1, one of adhesion molecules during vascular inflammation in endothelial cells, we first investigated the effect of TNF-α on the VCAM-1 expression in the HUVECs (Fig. 1). As shown in Fig. 1A, VCAM-1 was not expressed in the basal condition. However, treatment of TNF-α induced VCAM-1 expression in a dose dependent manner at the range of 1~30 ng/ml. VCAM-1 expression in the HUVECs reached a maximum at 15 ng/ml of TNF-α. Moreover, TNF-α induced VCAM-1 expression in the endothelial cells within 6 hours (Fig. 1B). In order to study that TNF-α induces monocyte adhesion as a model for vascular inflammation in endothelial cells, BCECF-AM-labeled U937 cells were co-incubated with TNF-α-activated endothelial cells, and BCECF-AM-labeled U937 cells were found to be firmly adhered on the surface of endothelial cells when the endothelial cells were incubated with TNF-a (15 ng/ml) for 18 hour (Fig. 1C).

The effect of MAPKs inhibitors on the VCAM-1 and monocyte adhesion

In mammalian cells, MAPKs are strongly activated by growth factors, environmental stresses and inflammatory cytokines (Cybulsky et al, 2001). To study the effect of TNF-α on the MAPKs (ERK, p38 MAPK, and JNK), we investigated the phosphorylation (active form) by Western blot. As shown in Fig. 2A, all types of MAPKs (ERK, p38 MAPK, and JNK) in the endothelial cells were activated by the treatment of TNF-α. Since the role of TNF-α in the activation of MAPKs was established, we examined which types of MAPKs are involved in TNF-α-induced VCAM-1 expression and monocyte-endothelial adhesion in the endothelial cells. Thus, we compared the effect of specific inhibitors of MAPKs on TNF-α-induced VCAM-1 expression using Western blotting. As shown in Fig. 2B, 10 mmol/L of SB203580, an inhibitor of p38 MAPK, significantly re-

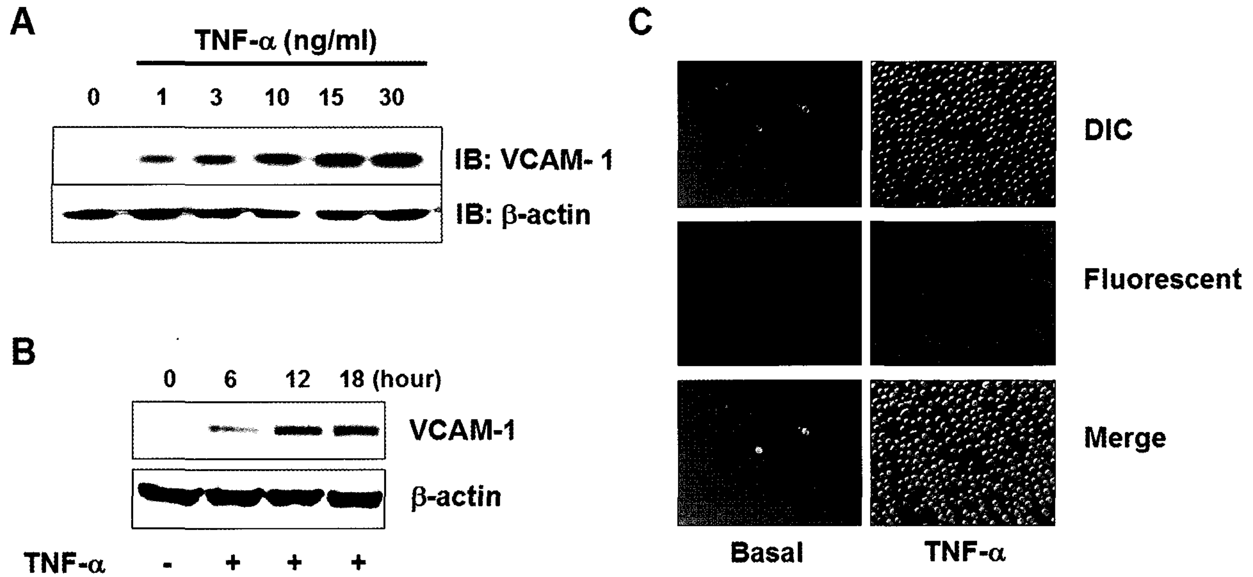

Fig. 1. Tumor necrosis factor-α (TNF-α) induced the vascular cell adhesion molecule-1 (VCAM-1) and monocyte adhesion on the endothelial cells. (A) TNF-α induced the expression of VCAM-1 in a dose dependent manner at the range of 1~30 ng/ml. Expression level of VCAM-1 was determined with immonoblotting for specific anti-VCAM-1. β-actin: loading control. (B) Time-dependent change of VCAM-1 induced by TNF-α in the endothelial cells. (C) Effect of TNF-α on the monocyte adhesion on the endothelial cells. Monocyte adhesion assay was performed as described in Methods.

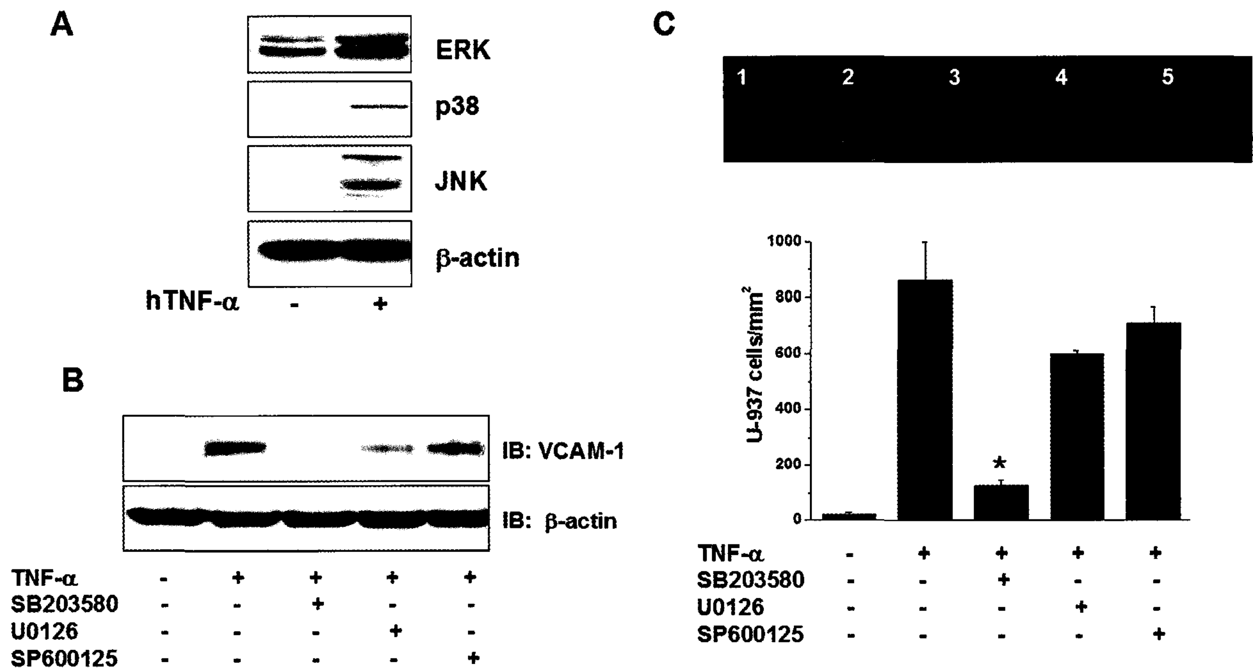

Fig. 2. p38 MAPK is involved in the TNF-α-induced VCAM-1 and monocyte adhesion. (A) Effect of TNF-α on the ERK, p38, and JNK activation in the human endothelial cells. Phosphorylation of ERK, p38 and JNK were determined with immunoblotting. β-actin : loading control. (B) Effect of MAPK inhibitors on the VCAM-1 expression in the human endothelial cells. β-actin : loading control. (C) Effect of MAPK inhibitors (10 μM SB203580, a p38 inhibitor; 40 μM U0126, an ERK inhibitor; 10 μM SP600125, a JNK inhibitor) on the monocyte adhesion to the endothelial cells. Monocyte adhesion assay was performed as described in Methods. Values of monocyte adhesion represent U937 cell number per mm^2 for 3 separate experiments.

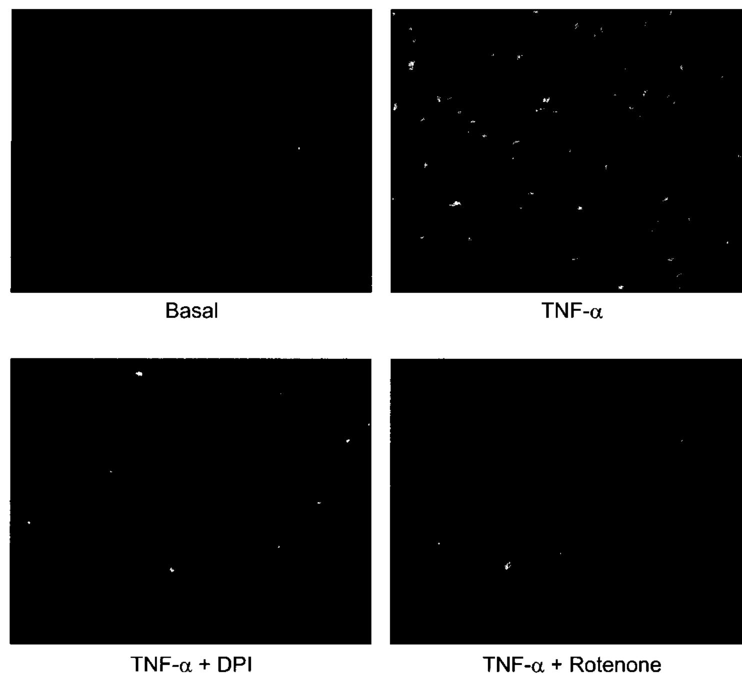

Fig. 3. NADPH oxidase and mitochondrial ROS are involved TNF-α-induced superoxide production in the endothelial cells. TNF-α induced the superoxide production in the endothelial cells. The superoxide production was determined by dihydroethidine staining and visualized with confocal microscopy as described in Methods.

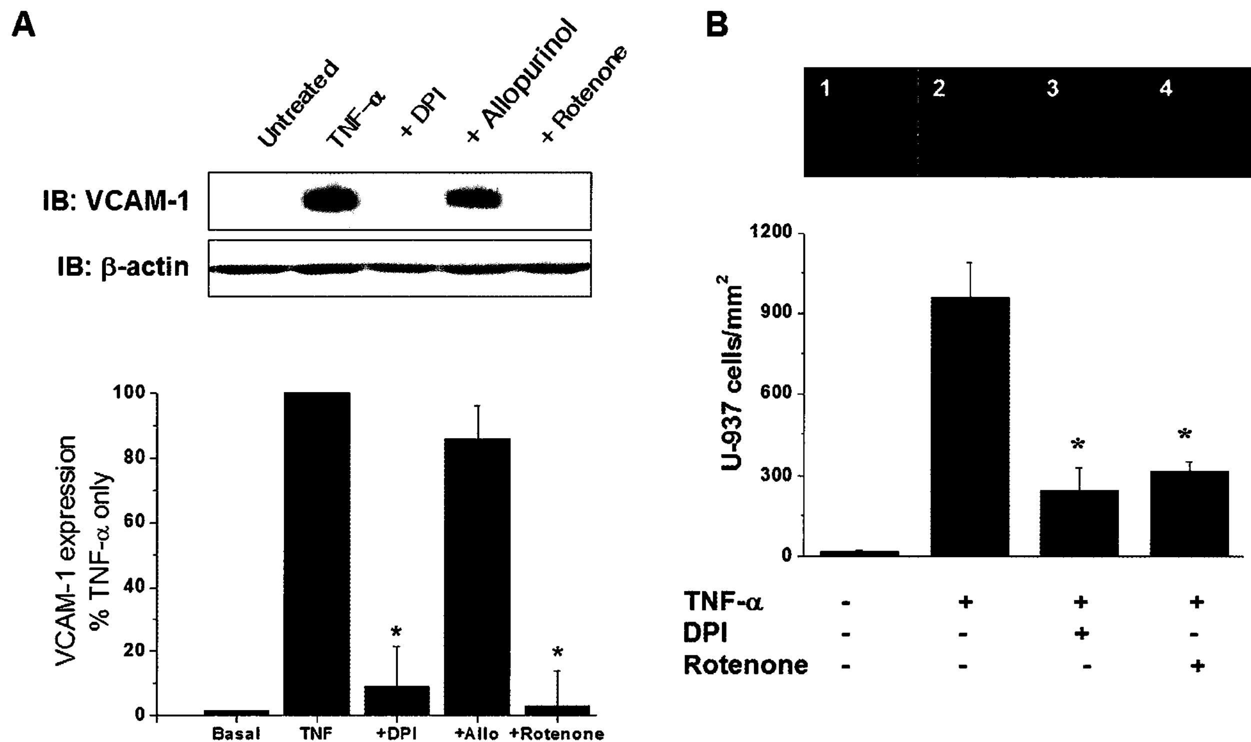

Fig. 4. NADPH oxidase and mitochondrial ROS are involved in TNF-α-induced VCAM-1 and monocyte adhesion in the endothelial cells. (A) Effect of several oxidase inhibitors on the TNF-α-induced VCAM-1 in the endothelial cells. Diphenyleneiodonium (DPI, 10 μM), an inhibitor of NADPH oxidase; allopurinol (10 μM), an inhibitor of xanthine oxidase; rotenone (10 μM), an inhibitor of mitochondrial oxidase. Densitometric data were plotted at bottom. Data are mean±SE for 3 separate experiments. (B) Effect of DPI and rotenone on the TNF-α-induced monocyte adhesion in the endothelial cells. Densitometric data were plotted at bottom. Values of monocyte adhesion represent U937 cell number per mm2 for 3 separate experiments.

μmol/L of U0126). Also, SB203580 significantly inhibited the TNF- α-induced monocyte adhesion in HUVEC. Taken together, our data suggest that mainly p38 MAPK is involved in TNF- α-induced VCAM-1 and monocyte adhesion in HUVEC.

TNF- α activated NADPH oxidase and mitochondrial oxidase in the HUVECs

To study the source of ROS in the TNF- α-treated HUVECs, we investigated the effect of specific oxidase inhibitors on superoxide production in the TNF- α-treated HUVECs, by measuring the change of superoxide production with dihydroethidine, fluorescent dye detecting superoxide anion, as described in Methods. As shown in Fig. 3, fluorescent signal for superoxide anion was minimal in the basal condition, however, treatment of TNF- α for 18 hours induced two types of fluorescent signal in the dihydroethidine-loaded endothelial cells. One strong signal was detected in nucleus and the other was detected as a dot pattern in cytoplasm, suggesting mitochondrial ROS. Diphenyleneiodonium (DPI, 10 μmol/L), an inhibitor of NADPH oxidase, inhibited TNF- α-induced superoxide production, especially nuclear fluorescent signal, whereas rotenone (1 μmol/L), an inhibitor of mitochondrial complex I, inhibited TNF- α-induced superoxide production, especially dotted fluorescent signal in cytoplasm. This data suggest the involvement of NADPH oxidase and mitochondrial oxidase in the TNF- α-induced superoxide production in the endothelial cells.

NADPH oxidase and mitochondrial ROS were involved in monocyte adhesion and VCAM-1 expressio in TNF- α-treated HUVECs

Based on the above results, we investigated the role of NADPH oxidase and mitochondrial ROS in the VCAM-1 and monocyte adhesion in the endothelial cells. Diphenyleneiodonium (DPI, 10 μmol/L), an inhibitor of NADPH oxidase, and rotenone (1 μmol/L), an inhibitor of mitochondrial complex I, significantly inhibited VCAM-1 expression in HUVECs induced by TNF- α. However, allopurinol (10 μmol/L), an inhibitor of xanthine oxidase, did not affect TNF- α-induced VCAM-1 expression (Fig. 4A). Furthermore, DPI or rotenone also suppressed TNF- α-stimulated monocyte adhesion in the endothelial cells by about 60～70% (Fig. 4B).

DISCUSSION

In the present study, we demonstrated that NADPH oxidase and mitochondrial ROS are involved in the TNF- α induced vascular inflammation in the endothelial cells. As a vascular inflammation model, we used to study monocyte adhesion model as described in previous report (Kim et al, 2006a).

Atheriosclerosis is known as vascular inflammatory disorder (Ross, 1999). The induction of inflammatory adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), and consequent monocyte adhesion to the lesion-prone areas seem to be the earliest visible markers which lead to atherosclerotic plaque development (Endres et al, 1997; Ross, 1999; Cybulsky et al, 2001; Libby et al, 2002). Expression of adhesion molecules accelerates the adhesion and migration of monocytes toward sites of inflammation in response to a variety of stimuli. Particularly, cellular adhesion molecules are an important component in atherosclerosis and the response to vascular injury (Huo & Ley, 2001). Histological studies demonstrate increased endothelial expression of VCAM-1 and intracellular adhesion molecule-1 (ICAM-1) in developing and established atherosclerotic lesions (Poston et al, 1992; Li et al, 1993). Early atherosclerotic events and the initiation of lesion formation appear to depend particularly on VCAM-1 (Cybulsky et al, 2001), and Dansky and colleagues suggested a major role of VCAM-1 played in the initiation of atherosclerotic process in $Vcam1^{D4D/D4D}Apoe^{-/-}$ mice (Dansky et al, 2001), likely reflecting an important function of VCAM-1 in recruitment of monocytes to the arterial intima. Based on the backgrounds, we investigated VCAM-1 to elucidate underlying mechanism for monocyte adhesion in the endothelial cells.

TNF- α is an inflammatory cytokine that is expressed by macrophages and several tissues (Sack, 2002). Vascular activation by TNF- α results in endothelial dysfunction following by vascular inflammation. TNF- α can activate MAPKs in the signaling pathway leading to expression of several cytokines (Baud & Karin, 2001). In the present study, even though TNF- α activated 3 types of MAPKs, only the inhibition of p38 MAPK reduced TNF- α-induced VCAM-1 expression and monocyte adhesion in the endothelial cells (Fig. 2), suggesting that the activation of p38 MAPK contributed to the TNF- α-induced vascular inflammation.

Since part of the signal transduction pathway that regulates the activation of VCAM-1 expression is redox-sensitive, the compounds with antioxidant properties are expected to have inhibitory effects on VCAM-1 expression (Fruebis et al, 1999). Generally stimuli that result in oxidative stress employ a number of cellular ROS generating systems, and the regulation of endothelial cell adhesion molecule expression, particularly VCAM-1 expression, is mediated by oxidation-reduction (redox)-coupled signaling mechanisms (Weber et al, 1994).

ROS can be produced in the vasculature from several sources, including NAD(P)H oxidases, mitochondrial oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (Griendling et al, 2000). In the present study, TNF- α induced superoxide production in the endothelial cells via activation of NADPH oxidase and mitochondrial oxidase (Fig. 3). Even though some evidence indicate that NAD(P)H oxidase is the major source of ROS in vascular endothelial cells (Griendling et al, 2000), mitochondrial ROS may also contribute to the TNF- α-induced vascular inflammation, since rotenone, an inhibitor of mitochondrial oxidase, suppressed TNF- α-induced superoxide generation, TNF- α-induced VCAM-1 and monocyte adhesion in the endothelial cells.

Taken together, our data suggest that p38 MAPK and ROS are involved in TNF- α-induced VCAM-1 expression and monocyte adhesion in the endothelial cells. Especially, TNF- α-induced ROS production was related closely with NADPH oxidase and mitochondrial ROS in endothelial cells. Considering the biological functions of vascular inflammation presented in these studies, it is quite possible that targeted molecules might contribute to the prevention of cell damage in inflammation and atherosclerosis.

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