Original Article

cAMP-Response Element-Binding Protein Mediates Tumor Necrosis Factor-α-Induced Vascular Cell Adhesion Molecule-1 Expression in Endothelial Cells

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Hypertension causes endothelial dysfunction, which plays an important role in atherogenesis. The vascular cell adhesion molecule-1 (VCAM-1) contributes to atherosclerotic lesion formation by recruiting leukocytes from blood into tissues. Tumor necrosis factor-α (TNFα) induces endothelial dysfunction and VCAM-1 expression in endothelial cells (ECs). We examined whether the cAMP-response element binding protein (CREB), a transcription factor that mediates cytokine expression and vascular remodeling, is involved in TNFα-induced VCAM-1 expression. TNFα induced phosphorylation of CREB with a peak at 15 min of stimulation in a dose-dependent manner in bovine aortic ECs. Pharmacological inhibition of p38 mitogen-activated protein kinase (p38-MAPK) inhibited TNFα-induced CREB phosphorylation. Adenovirus-mediated overexpression of a dominant-negative form of CREB suppressed TNFα-induced VCAM-1 and *c-fos* expression. Although activating protein 1 DNA binding activity was attenuated by overexpression of dominant negative CREB, nuclear factor-κB activity was not affected. Our results suggest that the p38-MAPK/CREB pathway plays a critical role in TNFα-induced VCAM-1 expression in vascular endothelial cells. The p38-MAPK/CREB pathway may be a novel therapeutic target for the treatment of atherosclerosis. (*Hypertens Res* 2006; 29: 39–47)

***Key Words*: endothelial factors, cytokine, gene expression, mitogen-activated protein kinase, signal transduction**

Introduction

The initial step of atherogenesis involves attachment of mononuclear leukocytes to endothelial cells (ECs) and migration into the subendothelial space (*1*). Adhesion molecules expressed in ECs play an important role in the attachment of mononuclear leukocytes. Various cardiovascular risk factors including hypertension have been shown to increase the levels of soluble adhesion molecules, such as the vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin (*2–4*). Carotid intima-media thickness has been positively correlated with the plasma level of circulating soluble cellular adhesion molecules (*5*). VCAM-1 is expressed in ECs predisposed to atherosclerotic lesion formation (*6*) and contributes to recruitment of mono-

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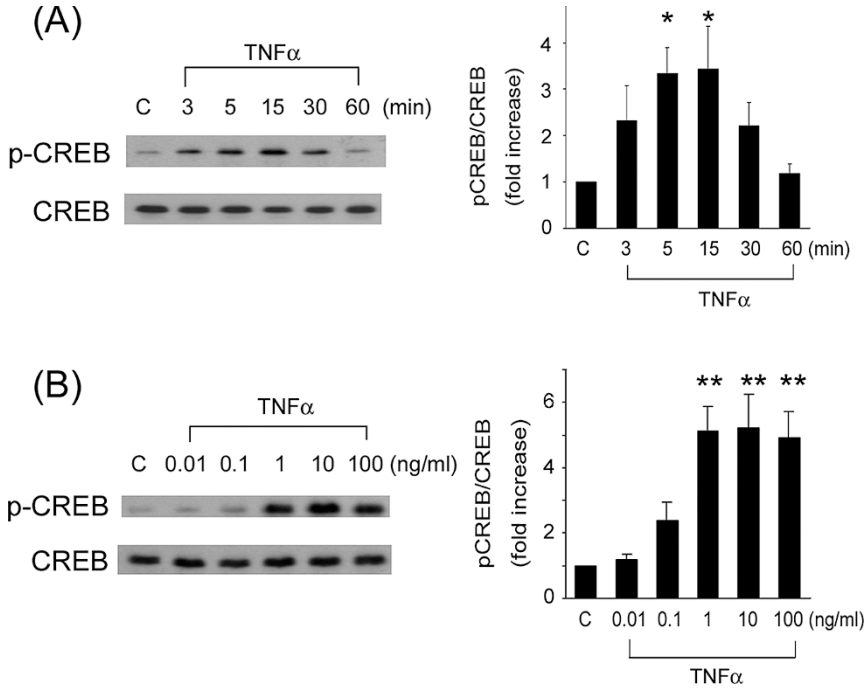

Fig. 1. *CREB is phosphorylated at Ser133 by TNFα. A: Bovine ECs were stimulated with TNFα (1 ng/ml) for varying periods indicated in the figure (*n = *4). B: Bovine ECs were stimulated with TNFα for 15 min at concentrations varying from 0.01 to 100 ng/ml (*n = *4). Phosphorylation of CREB was detected by Western blot analysis using a phospho-specific CREB antibody. The density of the specific band was scanned and quantified with an imaging analyzer. The ratio of phosphorylated CREB to total CREB in TNFα-stimulated cells is shown as the relative fold increase compared with that in unstimulated cells. The values are expressed as the mean ± SEM.* $^{*}p < 0.05$, $^{**}p < 0.01$ vs. *the control.*

nuclear leukocytes by binding to α4β1-integrin expressed on leukocytes (*7*).

Tumor necrosis factor-α (TNFα) is a multifunctional cytokine produced by activated macrophages, monocytes and lymphocytes. The vascular EC is an important target of TNFα (*1*, *8*). A previous study demonstrated that *in vivo* blockade of TNFα accelerated functional endothelial recovery after angioplasty (*9*). TNFα is known to modulate the expression of many genes involved in cytoadhesion, thrombosis, and inflammatory response in ECs, resulting in the acquisition of new functional capacities leading to atherosclerosis (*10*). VCAM-1 is one of the molecules induced by TNFα (*11*).

cAMP-response element (CRE)-binding protein (CREB) is a 43 kD nuclear transcription factor belonging to the CREB/ATF family (*12*, *13*). Phosphorylation of the serine residue at 133 (Ser133), which recruits a transcriptional coactivator, CREB-binding protein (CBP) or p300, is necessary for transcriptional activation. The phosphorylation of Ser133 is mediated by a variety of protein kinase pathways, such as 1) protein kinase A (PKA), 2) Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II (*14*), 3) extracellular signal-regulated protein kinase (ERK) (*15*, *16*), 4) p38 mitogen-activated protein kinase (p38-MAPK) (*17*), and 5) phosphatidylinositol 3-kinase (PI3-K) (*18*).

Although TNFα is known to activate transcription factors such as activating protein 1 (AP-1) and nuclear factor-κB (NF-κB) (*19*, *20*), it has not been examined whether TNFα activates CREB in ECs. We investigated whether CREB is activated by TNFα in bovine ECs. We report in the present study that TNFα phosphorylated CREB through p38-MAPK and CREB mediated TNFα-induced VCAM-1 expression.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL (Gaithersburg, USA). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, USA). Ionomycin, KN93 and SP60125 were purchased from Sigma Chemical Co. (St. Louis, USA). Recombinant human TNFα was a gift from Dainippon Pharmaceutical Co. (Osaka, Japan). PD98059 and wortmannin were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, USA). SB203580 and FR167653, inhibitors of p38-MAPK, were gifts from GlaxoSmithKline and Fujisawa Pharmaceutical Co. (Osaka, Japan), respectively. H89 was purchased from Seikagaku Co. (Tokyo, Japan). Horseradish peroxidase conjugated second antibodies (anti-rabbit or anti-mouse IgG) were purchased from VECTOR Laboratories Inc.

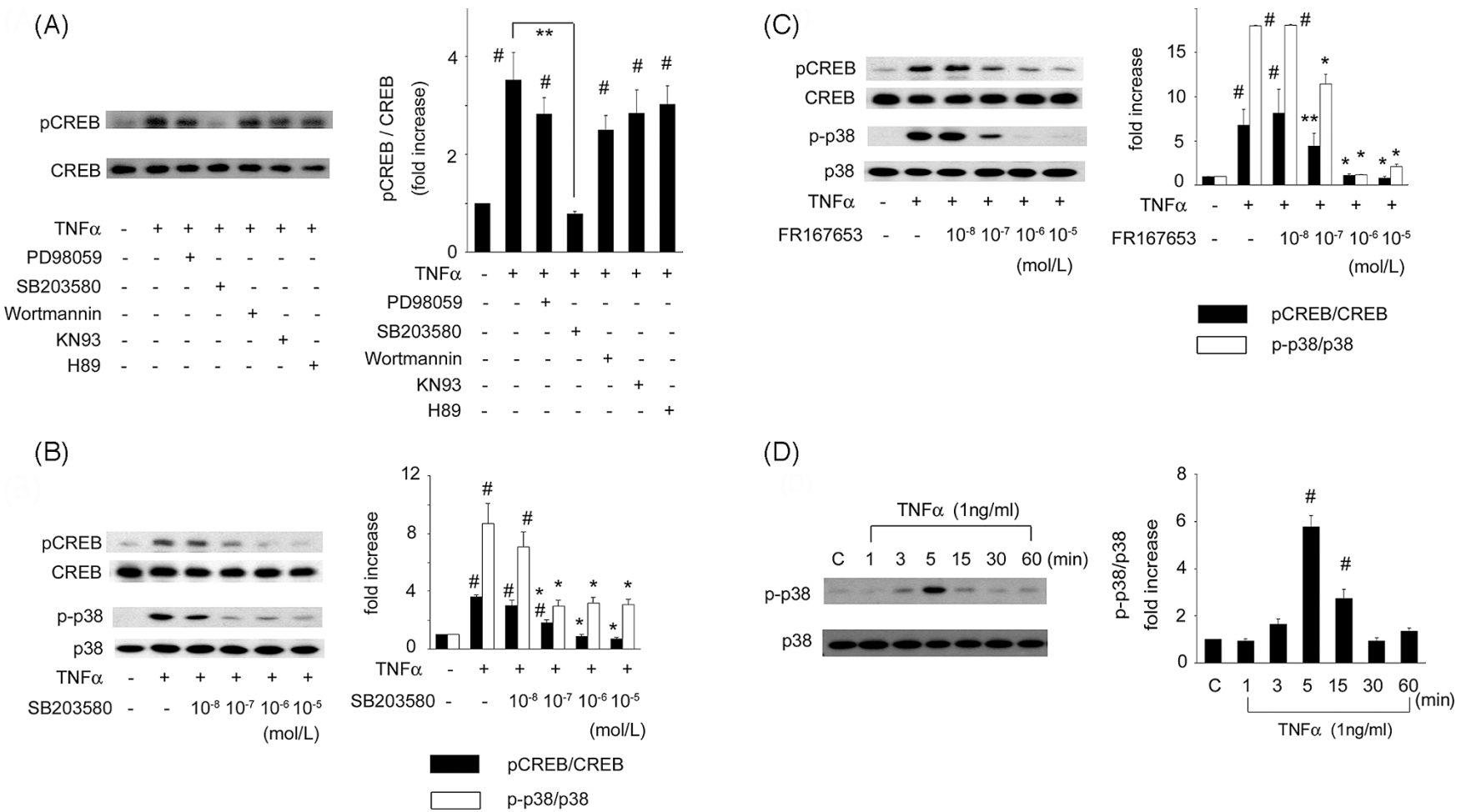

Fig. 2. *The p38-MAPK pathway mediates TNFα-induced CREB phosphorylation. A: Bovine ECs were preincubated with PD98059 (10 μmol/l), SB203580 (10 μmol/l), wortmannin (50 nmol/l), KN93 (10 μmol/l) or H89 (1 μmol/l) for 30 min and stimulated with TNFα (1 ng/ml) for 15 min. Phosphorylation of CREB was detected and analyzed as described in the legend to Fig. 1 (*n = *3). The ratio of phosphorylated CREB to total CREB in TNFα-stimulated cells is shown as the relative fold increase compared with that in unstimulated cells. The values are expressed as the mean ± SEM.* $^{**}p < 0.01$ *vs. TNFα,* $^{\#}p < 0.01$ *vs. the control. B, C: Bovine ECs were preincubated with (B) SB203580 or (C) FR167653 at concentrations varying from* 10^{-8} *to* 10^{-5} *mol/l for 30 min and stimulated with TNFα (1 ng/ml) for 15 min. D: Bovine ECs were stimulated with TNFα (1 ng/ml) for varying periods indicated in the figure. Phosphorylation of CREB and p38-MAPK was detected and analyzed as described in the legend to Fig. 1 (*n = *4–6). The ratio of phosphorylated CREB or p38-MAPK to total CREB or p38-MAPK in TNFα-stimulated cells is shown as the relative fold increase compared with that in unstimulated cells. The values are expressed as the mean ± SEM.* $^{\#}p < 0.01$ *vs. the control,* $^{*}p < 0.01$, $^{**}p < 0.05$ *vs. TNFα.*

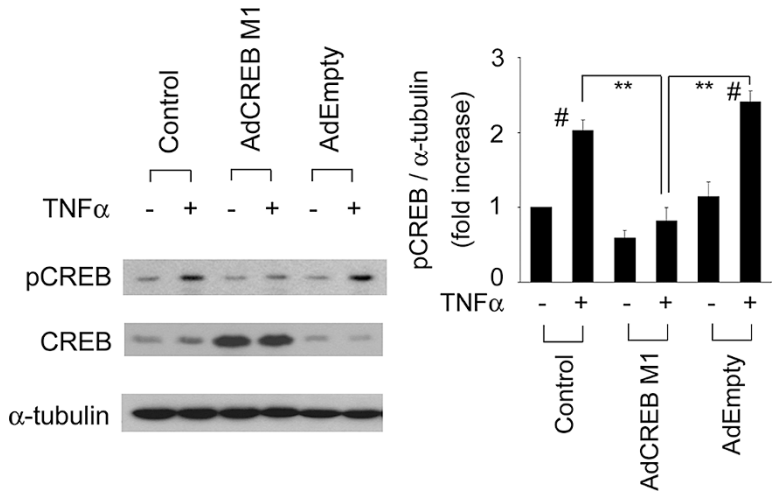

Fig. 3. *AdCREB M1 inhibits TNFα-induced CREB phosphorylation. Bovine ECs were infected with AdCREB M1 (30 MOI) or AdEmpty (30 MOI) and stimulated with or without TNFα (1 ng/ml) for 15 min. TNFα-induced CREB phosphorylation was detected by Western blot analysis (*n = *4). The ratio of phosphorylated CREB to α-tubulin in TNFα-stimulated cells is shown in the right panel as the relative fold increase compared with that in unstimulated cells. The values are expressed as the mean ± SEM.* **p < *0.01* vs. *AdCREB M1 TNFα (+),* #p < *0.01* vs. *control TNFα (–) or AdEmpty TNFα (–).*

(Burlingame, USA). Other antibodies used in the experiments were obtained from Cell Signaling Technology (Danvers, USA). Other chemical reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless specifically mentioned.

Cell Culture

The bovine aortic ECs were the gift of Katsuya Hirano (Kyushu University Graduate School of Medical Sciences) and grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C in DMEM with 10% FBS. Passages between 5 and 12 were used for the experiments. The investigation conformed with the Guide for the Care and Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Western Blot Analysis

Bovine ECs were lysed in a sample buffer (5 mmol/l EDTA, 10 mmol/l Tris-HCl, pH 7.6, 1% Triton X-100, 50 mmol/l NaCl, 30 mmol/l sodium phosphate, 50 mmol/l NaF, 1% aprotinin, 0.5% pepstatin A, 2 mmol/l phenylmethylsulfonyl fluoride and 5 mmol/l leupeptin). Western blot analyses of CREB, p38-MAPK and VCAM-1 were performed as described previously (*21*).

Adenovirus Vector Expressing a Dominant Negative Form of CREB

A recombinant adenovirus vector expressing a mutant of CREB (AdCREB M1) (*22*) in which the phosphorylation site at Ser133 was changed to alanine was a gift from Anthony J. Zeleznik (University of Pittsburgh, Pittsburgh, USA). Confluent bovine ECs were washed 2 times with PBS and incubated with AdCREB M1 or adenovirus empty vector (AdEmpty) under gentle agitation for 2 h at room temperature. Then the cells were washed 3 times, cultured in DMEM with 10% FBS for 2 days and used for the experiments. The multiplicity of infection (MOI) value indicates the number of viruses per cell added to a culture dish.

Northern Blot Analysis

Total RNA was prepared according to an acid–guanidinium–thiocyanate–phenol–chloroform extraction method. Northern blot analysis of *c-fos*, VCAM-1 and 18S rRNA was performed as described previously (*21*). The radioactivity of hybridized bands of *c-fos* and VCAM-1 mRNA, and 18S rRNA was quantified with a MacBAS Bioimage Analyzer (Fuji Film Co., Tokyo, Japan).

Preparation of Nuclear Extracts and Gel Mobility Shift Assay

The preparation of nuclear extracts and gel mobility shift assay were performed as described previously (*23*). DNA probes of AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) and NF-κB (5′-AGATGAGGGGACTTTCCCAGGC-3′) were end-labeled with ^{32}P γ-ATP. Ten micrograms of nuclear extracts were incubated with 1×10^5 cpm of labeled DNA probe for 30 min at room temperature and electrophoresed on 4% acrylamide gel. A fifty-fold molar excess of unlabeled DNA was added as a competitor. After electrophoresis, the

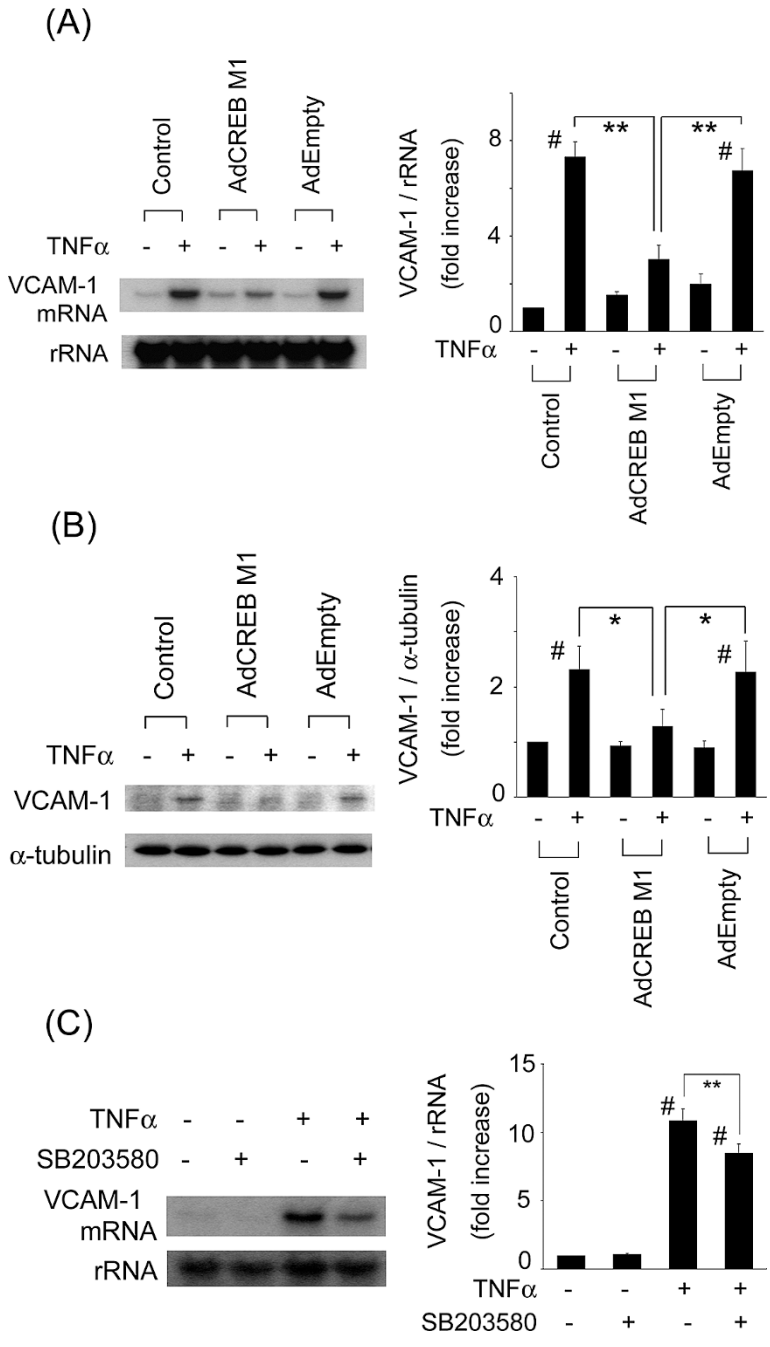

Fig. 4. *AdCREB M1 inhibits TNFα-induced VCAM-1 mRNA and protein expression. A: Bovine ECs were infected with AdCREB M1 (30 MOI) or AdEmpty (30 MOI) and stimulated with or without TNFα (1 ng/ml) for 4 h. TNFα-induced VCAM-1 mRNA expression was detected by Northern blot analysis and the radioactivities of the bands were measured with an imaging analyzer (*n = *4). The radioactivity of VCAM-1 mRNA in TNFα-stimulated cells was normalized against that of rRNA and shown as the relative fold increase compared with that in unstimulated cell. B: Bovine ECs were infected with AdCREB M1 (30 MOI) or AdEmpty (30 MOI) and stimulated with or without TNFα (1 ng/ml) for 12 h. TNFα-induced VCAM-1 protein expression was detected by Western blot analysis (*n = *4) and the ratio of VCAM-1 expression to α-tubulin in TNFα-stimulated cells is shown in the right panel as the relative fold increase compared with that in unstimulated cells. C: Bovine ECs were preincubated with SB203580 (10 μmol/l) for 30 min and stimulated with TNFα (1 ng/ml) for 4 h. TNFα-induced VCAM-1 mRNA expression was detected by Northern blot. The values are expressed as the mean±SEM.* $^{**}p < 0.01$ vs. *AdCREB M1 TNFα (+) or TNFα,* $^{*}p < 0.05$ vs. *AdCREB M1 TNFα (+),* $^{\#}p < 0.01$ vs. *control TNFα (–) or AdEmpty TNFα (–).*

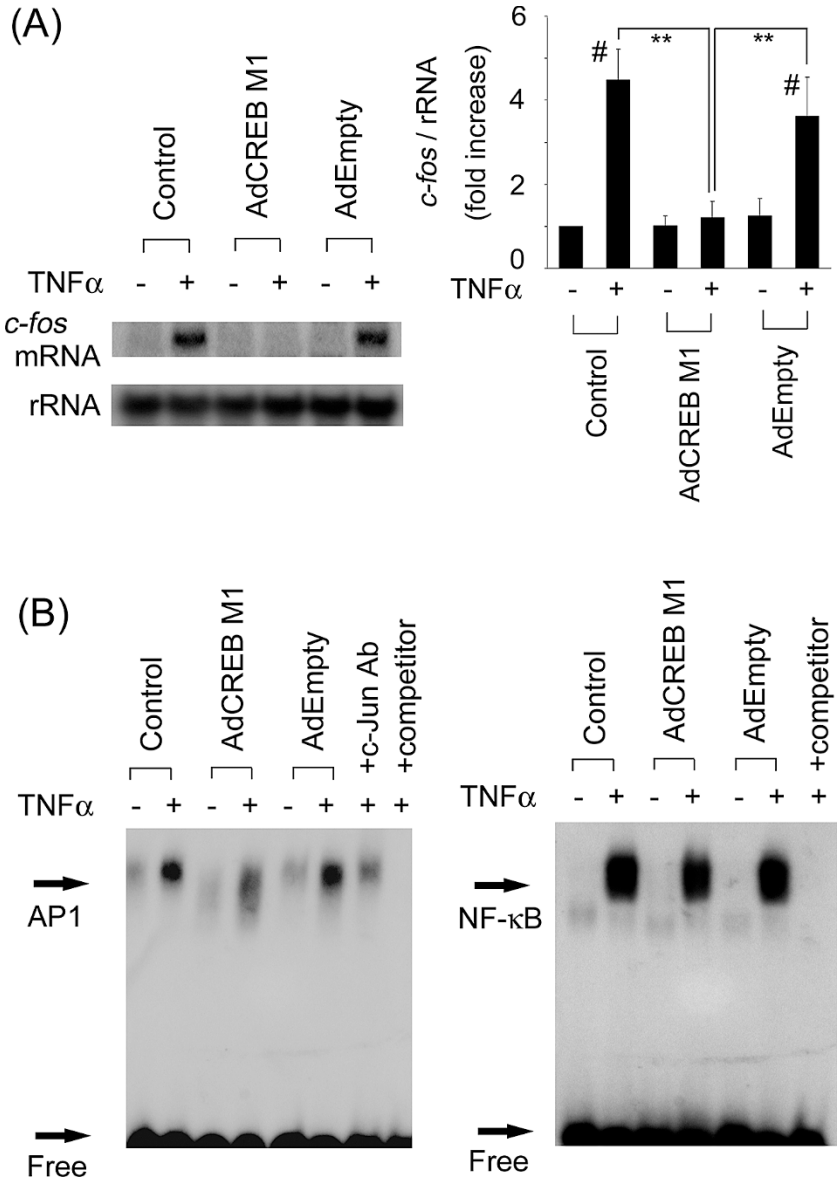

Fig. 5. *AdCREB M1 inhibits TNFα-induced* c-fos *mRNA expression and AP-1 DNA binding activity. A: Bovine ECs were infected with AdCREB M1 (30 MOI) or AdEmpty (30 MOI) and stimulated with or without TNFα (1 ng/ml) for 30 min. TNFα-induced* c-fos *mRNA expression was detected by Northern blot analysis and the radioactivities of the bands were measured with an imaging analyzer (*n = *4). The radioactivity of* c-fos *mRNA was normalized against that of rRNA. The ratio in TNFα-stimulated cells is shown as the relative fold increase compared with that in unstimulated cells. The values are expressed as the mean±SEM.* **$p < 0.01$ vs. *AdCREB M1 TNFα (+),* #$p < 0.01$ vs. *control TNFα (–) or AdEmpty TNFα (–). B: Bovine ECs were infected with AdCREB M1 (30 MOI) or AdEmpty (30 MOI) and stimulated with or without TNFα (1 ng/ml) for 4 h. Nuclear extracts were prepared and incubated with radiolabeled AP-1 (left panel) or NF-κB (right panel) probe for 30 min and electrophoresed. A fifty-fold molar excess of unlabeled probe was used as a competitor. For the supershift assay, an antibody against c-Jun was added to the binding reaction mixtures. The same results were obtained in other independent experiments and a representative autoradiogram is shown (*n = *4).*

gels were dried and exposed to X-ray films.

Statistical Analysis

Statistical analysis was performed with 1-way ANOVA and Fisher's test if appropriate. Values of $p<0.05$ were considered to indicate statistical significance. Data are shown as the mean±SEM.

Results

Phosphorylation of CREB at Ser133 by TNFα

To examine whether CREB is phosphorylated in response to TNFα, we performed Western blot analysis using an antibody that only recognizes the phosphorylated form of CREB at Ser133 (p-CREB). TNFα stimulated phosphorylation of CREB with a peak at 15 min of stimulation (Fig. 1A). TNFα dose-dependently increased phosphorylation of CREB at 15 min of stimulation (Fig. 1B).

The p38-MAPK Pathway Mediates TNFα-Induced CREB Phosphorylation

Several protein kinases are reported to phosphorylate CREB. We examined which pathway is responsible for TNFα-induced CREB phosphorylation. SB203580 (10 μmol/l), a p38-MAPK inhibitor, completely blocked TNFα-induced CREB phosphorylation (Fig. 2A). PD98059 (10 μmol/l), an ERK kinase (MEK) inhibitor, wortmannin (50 nmol/l), an inhibitor of PI3-K, KN93 (10 μmol/l), an inhibitor of CAMKII, and H89 (1 μmol/l), an inhibitor of PKA, did not affect TNFα-induced CREB phosphorylation (Fig. 2A). SP600125, a *c-jun* N-terminal kinase inhibitor, also had no effect on TNFα-induced CREB phosphorylation (data not shown). SB203580 was first described as an inhibitor of p38-MAPK activity that acts by competing with ATP for binding; however, it was later demonstrated that SB203580 also prevents p38-MAPK phosphorylation/activation (*24–26*). SB203580 dose-dependently inhibited TNFα-induced CREB and p38-MAPK phosphorylation (Fig. 2B). To confirm the role of p38-MAPK, we used another p38-MAPK inhibitor, FR167653. FR167653 dose-dependently inhibited TNFα-induced CREB and p38-MAPK phosphorylation (Fig. 2C). TNFα stimulated phosphorylation of p38-MAPK with a peak at 5 min of stimulation, which is faster than phosphorylation of CREB (Fig. 2D). PD98059 and wortmannin at the same concentrations used in Fig. 2 inhibited TNFα-induced ERK and Akt (a target molecule of PI3-K) activation, respectively (data not shown). KN93 and H89 at the same concentrations also inhibited ionomycin- and forskolin-induced CREB phosphorylation, respectively (data not shown). Therefore, the concentrations of these protein kinase inhibitors were sufficient. These data suggest that the p38-MAPK pathway is critical for TNFα-induced CREB phosphorylation.

Overexpression of a Dominant Negative Form of CREB Inhibits TNFα-Induced VCAM-1 Expression

To clarify the role of CREB in the TNFα signaling, we overexpressed a dominant negative form of CREB by an adenovirus vector (AdCREB M1). We used AdEmpty as a negative control for the infection of adenovirus. Phosphorylation of CREB by TNFα was attenuated by infection of AdCREB M1, but not by AdEmpty (Fig. 3). A previous study demonstrated that TNFα stimulated VCAM-1 expression in ECs (*11*). In the present study, AdCREB M1 but not AdEmpty suppressed TNFα-induced VCAM-1 mRNA and protein expression (Fig. 4A, B). SB203580 also suppressed TNFα-induced VCAM-1 mRNA expression (Fig. 4C), suggesting that the p38-MAPK/CREB pathway plays an important role. It is known that TNFα induces VCAM-1 expression through activation of NF-κB and AP-1 (*27*). AP-1 is a heterodimer of c-Fos and c-Jun and CRE is one of the important *cis*-DNA elements regulating *c-fos* gene expression. We therefore hypothesized that dominant negative CREB may affect *c-fos* induction and AP-1 activation. AdCREB M1 but not AdEmpty suppressed TNFα-induced *c-fos* mRNA expression (Fig. 5A). Furthermore, AdCREB M1 suppressed AP-1 DNA binding activity to the consensus sequence induced by TNFα, but it did not affect NF-κB binding activity (Fig. 5B). The binding of AP-1 was specific because the band was eliminated by a 50 mol excess of unlabeled competitor, and the band was supershifted by addition of an antibody against c-Jun. These data suggest that AdCREB M1 may suppress TNFα-induced VCAM-1 gene expression through inhibition of not only CREB but also AP-1 activity.

Discussion

In the present study, we showed that TNFα activated CREB through p38-MAPK. Inhibition of CREB function by a dominant negative molecule suppressed TNFα-induced AP-1 activity and VCAM-1 expression.

The results of a search for *cis*-DNA elements of the VCAM-1 gene promoter by TFSEACH showed the presence of a possible CRE site in the promoter of VCAM-1 at −1686 bp. Therefore, our result suggests that the CRE site of the VCAM-1 gene promoter may play an important role in VCAM-1 expression induced by TNFα. A previous study demonstrated that TNFα stimulated VCAM-1 expression through two NF-κB sites (present at −63 bp and −77 bp from the transcription initiation site) (*11*). Ahmad *et al*. reported that the AP-1/NF-κB complex was induced by TNFα and regulated VCAM-1 gene expression (*27*). AP-1 can interact with other transcription factors and modulate their transcriptional activity (*28*).The p65 subunit of NF-κB requires a co-factor protein for transcriptional activity and can interact with c-Fos and c-Jun through the Rel homology domain (*29*). CRE in the promoter region of the *c-fos* gene plays an important role in the induction of *c-fos* by many stimuli (*30–32*). We confirmed that CRE mediates *c-fos* expression by TNFα. These data suggest that inhibition of AP-1 activity by AdCREB M1 may be involved in the suppression of TNFα-induced VCAM-1 expression. However, further study is necessary to confirm the role of the AP-1 site of the VCAM-1 gene promoter in response to TNFα.

TNFα is known to activate the mitogen-activated protein kinases (MAPKs), such as *c-jun* NH_2-terminal kinase and p38-MAPK in ECs (*33*, *34*). A previous study demonstrated that p38-MAPK mediated actin filament reorganization by several stimuli, such as vascular endothelial growth factor or oxidative stress, in human umbilical vein ECs (*35*). Another study demonstrated that p38-MAPK negatively regulated cell survival and proliferation by FGF-2 stimulation in bovine capillary ECs (*36*). In the present study, we demonstrated that p38-MAPK mediated TNFα-induced CREB phosphorylation and could modulate the expression of cytoadhesion molecules. The p38-MAPK family includes four isoforms, p38α, p38β, p38γ and p38δ. Vascular EC expresses p38α, p38β and p38δ (*37*). SB203580 inhibits p38α and p38β, and thus p38α or p38β may mediate TNFα-induced CREB phosphorylation.

Atherosclerotic lesion progression has been shown to depend on persistent, chronic inflammation in the arterial wall and is characterized by the recruitment of monocytes and lymphocytes to the arterial wall (*38*). Adhesion molecules and chemotactic factors mediate the entry of the leukocytes into the subendothelial space. The first step in adhesion, the rolling of leukocytes along the endothelial surface, is mediated by selectins which bind to carbohydrate ligands on leucocytes (*39*, *40*). The firm adhesion of monocytes and T lymphocytes to endothelium is mediated by VCAM-1 on the endothelium, which interacts with the integrin VLA-4 on monocytes and T lymphocytes (*7*). Therefore, VCAM-1 is assumed to be important for atherosclerogenesis, and knockout strategies have been attempted. Although VCAM-1-null mice die during embryogenesis (*41*), it has been shown that atherosclerotic lesion was reduced that the size of atherosclerotic lesions is reduced in VCAM-1 domain 4-deficient mice (*42*), suggesting that VCAM-1 is indeed an important gene product directly involved in the formation of atherosclerotic lesions.

In the present study, we demonstrated the possible involvement of CREB in TNFα-induced VCAM-1 expression. In addition to TNFα, angiotensin II has been shown to stimulate VCAM-1 expression (*43*, *44*), and we and others previously reported that angiotensin II stimulated phosphorylation of CREB (*32*, *45*). Inhibition of CREB may suppress not only TNFα-induced but also angiotensin II-induced VCAM-1 expression. Furthermore, it was previously reported that high blood pressure activates MAPKs (*46–48*) and that p38-MAPK activation induced by high blood pressure is involved in endothelial dysfunction (*48*). Therefore, inhibition of the p38-MAPK/CREB pathway may attenuate endothelial dysfunction in patients with hypertension. Our data suggest that the p38-MAPK/CREB pathway could be a therapeutic target for the prevention of atherosclerosis.

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