Raf Kinase Inhibitor Protein (RKIP) Inhibits Tumor Necrosis Factor-α (TNF-α) Induced Adhesion Molecules Expression in Vascular Smooth Muscle Bells by Suppressing (Nuclear Transcription Factor-κB (NF-kappaB) Pathway

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Background: Raf kinase inhibitor protein (RKIP) regulates growth and differentiation and plays a role in key signal transduction cascades in mammalian cells. Nevertheless, the underlying mechanism for which RKIP regulates cell-cell adhesion remains unknown. Our study investigated the function of the RKIP overexpression on adhesion molecules expression induced by tumor necrosis factor (TNF)-α in cultured mouse vascular smooth muscle cells (MOVACs).

Material/Methods: The expression levels of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were detected by ELISA kit, reverse transcription-PCR, and western blot assays. The protein expression of RKIP, p65, and inhibitor of nuclear factor (NF)-κB (IκBα) were detected by western blot analysis. The activity of NF-kappaB was determined using a Dual-Luciferase Reporter assay.

Results: The results showed that MOVACs transfected with pCMV5-HA-RKIP significantly inhibited TNF-α induced mRNA and protein expression of ICAM-1 and VCAM-1. The adhesion of THP-1 cells was also detected and inhibited by pCMV5-HA-RKIP in TNF-α-treated MOVACs. RKIP also suppressed the TNF-α-induced activation of NF-κB and the protein expression of phosphorylated IκB-α, and promoted the protein expression of IκB-α and nuclear translocation of p65 NF-κB. Furthermore, RKIP and the inhibitor of NF-κB (BAY11-7082) reduced the upregulation of ICAM-1 and VACM-1 induced by TNF-α.

Conclusions: Taken together, these results suggested that RKIP may inhibit the TNF-α-induced expression of adhesion molecules in MOVACs through inactivation of the NF-κB pathway.

MeSH Keywords: Cell Adhesion Molecules • Phosphatidylethanolamine Binding Protein • Receptor Activator of Nuclear Factor-kappa B • Tumor Necrosis Factor-alpha

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Background

Raf kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine-binding protein family and is characterized by a small, conserved cytosolic protein originally purified from the bovine brain [1]. RKIP is involved in biosynthesis of the plasma membrane, neurodevelopment, spermatogenesis, apoptosis, and other physiological and pathological processes [2]. It has been reported that RKIP is also associated with an increasing number of diseases through its involvement with signal transduction pathways, including nuclear factor (NF)-kB/G protein-coupled receptor kinase signal transduction pathway and the Raf-1-mitogen-activated protein kinase signaling pathway [3]. A study by Wen et al. [4] showed that RKIP may be involved in Parkinson’s disease by interacting with cyclin-dependent kinase 5 in neurons.

The downregulation of RKIP has also been shown to be closely associated with several significant tumor behaviors, including proliferation, adhesion, and metastasis, and prognosis [5–7]. The overexpression of RKIP has been shown to promote macrophage differentiation by modulating NF-kB signaling, which suggests a link between RKIP and the expression of cell sub-stratum adhesion proteins [8]. The adhesion of cells to extracellular matrix (ECM) is a dynamic process, which is critical to the mechanical coupling of intracellular and extracellular environments required for cell migration [9]. Cellular adhesion is modulated predominantly by the activity of adhesion molecules in diverse endothelial cells and smooth muscle cells [10–12]. Cell adhesion proteins, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, on vascular smooth muscle cells are directly involved in inflammation and cardiovascular disease [13]. The activity of cell adhesion molecules has long been considered to be a direct consequence of their adhesive properties [14]; therefore, RKIP may be vital in cell migration, adhesion, and metastasis. A previous study reported that RKIP inhibits the migration and invasion of PC-3M human prostate cancer cells through regulation of ECM [15]. However, until now, there has been no clear evidence to show effect of RKIP on the activity of cell adhesion molecules or the underlying molecular mechanism in vascular smooth muscle cells.

Consequently, based on multiple lines of evidence, the present study investigated whether RKIP can affect the activity of adhesion molecules and aimed to determine the role of the NF-kappaB signaling pathway in this process in TNF-α-induced vascular smooth muscle cells.

Material and Methods

Cell culture and TNF-α treatment

The mouse vascular smooth muscle cells (MOVACs) were of the Movas-1 vascular smooth muscle cell line, which was derived from aortic smooth muscle cells of male C57BL6 mice (4–6-week-old; 20±2 g) supplied by the Laboratory Animal Center of Tianjin Nankai Hospital (Tianjin, China) as described by Golovina and Blaustein [16]. All experimental protocols were approved by the Ethics Committee of Nankai Hospital and were performed in accordance with the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in a humidity- and temperature-controlled room with food and water provided ad libitum. Light was controlled by a 12-hour light/dark cycle. Mice were acclimatized for more than three days before surgical procedures.

In brief, animals were anesthetized (pentobarbital, 50 mg/kg, ip) and pectoral muscles were dissected. The MOVACs were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in a humidified atmosphere in a 5% CO₂ incubator at 37°C until they reached confluence. Culture medium containing TNF-α (10 ng/mL) was then added to the cells and cultured for four hours at 37°C.

Plasmid transfection

To induce the overexpression of RKIP, the MOVACs (5×10⁵ cells/ml) were cultured as described until 80% confluent, following by 20 μg of either the pCMV5-HA-RKIP (RKIP) plasmid, which was constructed according to previous literature [17] or empty vector plasmid was transfected into the cells using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol and cultured for 24 hours at 37°C. The cells were harvested for analyzing the expression of RKIP or were treated with TNF-α for further experiments.

ELISA

The cell surface expression levels of ICAM-1 and VCAM-1 on the MOVAC monolayers were quantified using ELISA, according to the manufacture’s protocols of ICAM-1 and VCAM-1 mouse ELISA kits (Abcam, Cambridge, MA, USA). In brief, the MOVACs (2×10⁵ cells/ml) were seeded into a 96-well plate and treated with 10 ng/ml TNF-α and 20 μg pCMV5-HA-RKIP. The cells were harvested and incubated with mouse anti-ICAM-1 (1: 2,000) and rabbit anti-VCAM-1 monoclonal antibody (1: 5,000) from the ELISA kits at room temperature for one hour, followed by
adding TMB One-Step substrate reagent to each well and then incubating for 30 minutes. Finally, stop solution was added and the absorbance was read immediately at 450 nm.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis**

Total RNA was isolated from the MOVACs using an RNeasy Plus Universal kit (Qiagen, CA, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.). PCR analysis was performed using a SYBR Green Real-Time PCR Master Mix kit (Takara Biotechnology, Inc., Dalian, China). In brief, 45 μL reaction solution containing 50 ng cDNA, 25 μL Fast Start Universal SYBR Green Master, 0.5 μL forward primer and reverse primer and 19 μL PCR-grade water were subjected to 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds, 58°C for 60 seconds, and 68°C for 45 seconds. The expression levels of each gene were normalized to the expression of the control gene (GAPDH). The primer sequences for the RT-PCR analysis were as follows: ICAM-1, forward 5'-CTCAATGTCGAGGTTCG-3' and reverse 5'-CAGTGGGAAAG TGCCATCCT-3'; VCAM-1, forward 5'-CCAAGGATCCAGAATTCA-3' and reverse 5'-TAAGTGAAGGCGATTTCC-3'; GAPDH, forward 5'-GCTCTCACACCTAGGGA AA-3' and reverse 5'-AGGAG GCATTGCTGATGAC-3'. The expression levels of the genes were calculated using GAPDH mRNA as a control using the 2^(-ΔΔCq) method [18].

**Western blot analysis**

The cells were washed twice with PBS and harvested in a lysis buffer containing 1% SDS, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 20 mM EDTA. Total protein was extracted from the harvested cells using RIPA Buffer (Pierce; Thermo Fisher Scientific, Inc.). Nuclear proteins were extracted using NE-PER kits (Pierce; Thermo Fisher Scientific, Inc.). All proteins were quantified using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). For western blot analysis, ~30 μg protein was electrophoresed on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. The blots were blocked in 5% non-fat milk in PBS for one hour, and then incubated with rat monoclonal anti-ICAM-1 (1: 500), anti-VCAM-1 (1: 500), anti-RKIP (1: 2,000), rabbit polyclonal anti-p65 (1: 300), anti-inhibitor of NF-kappaBβ (κB-α;1: 250) or phosphorylated IκB-α (1: 1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies overnight at 37°C. The membrane was then incubated with horseradish-conjugated secondary antibodies for one hour. Peroxidase activity on the membrane was visualized on x-ray films using a standard enhanced chemiluminescence procedure. The immunoreactive bands were visualized using Immuno Star LD (Wako Pure Chemical, Osaka, Japan), and then measured using LAS-4000 Mini (Fuji Film Co., Ltd., Tokyo, Japan).

**Cell adhesion assay**

The adhesion of monocytes to the MOVACs was determined using THP-1 cells, as previously described [19,20]. The MOVACs transfected with pCMV5-HA-RKIP were cultured in a 96-well plate (2×10^4 cells/mL) until they reached confluence. TNF-α (10 ng/mL) was added and cultured for four hours at 37°C. The THP-1 cells (American Type Culture Collection, Manassas, VA, USA) were co-incubated with MOVACs for one hour at 37°C. The cells were washed three times with PBS to remove non-adherent cells. The cells bound to the MOVACs were added to a substrate solution consisting of 2 mM 3', 5', 5' tetramethylbenzidine in 0.1 M sodium acetate buffer (pH 4.2) containing 0.1% cetrimethyl ammonium bromide as a peroxidase solubilizing agent. The absorbance at 490 nm was then determined using an automated microplate reader (Spectramax 190, Molecular Devices LLC, Sunnyvale, CA, USA).

**NF-kappaB activity assay**

NF-kappaB activity was determined using a Dual-Luciferase reporter assay system (Promega Corporation, Madison, WI, USA). Briefly, the MOVACs were incubated in 6-well plates(5×10^5 cells/mL) and co-transfected with 1 μg NF-kappaB reporter plasmid and 100 ng control Renilla luciferase vector (pRL-TK; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. Following transfection, the cells were incubated for 10 minutes at room temperature and then cultured for five hours in complete culture medium. Following removal of Lipofectamine from the medium, 10 ng/mL of TNF-α was added, and the cells were harvested four hours later. The luciferase activity was measured in duplicate in each experiment using the Dual Luciferase reporter assay system. The firefly luciferase activity of NF-kappaB was normalized to the Renilla luciferase activity (Promega Corporation). The luciferase activities of samples containing the NF-kappaB responsive element were compared with the luciferase activities in samples transfected with the control plasmid, and reported as the fold increase.

**Statistical analysis**

The results were analyzed using one-way analysis of variance for multiple groups, followed by Bonferroni correction. For two-group comparison, Student’s unpaired t-test was performed on SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of the mean. A P<0.05 was considered to indicate a statistically significant difference. All results are representative of at least three independent experiments.
RKIP inhibits TNF-α-induced surface expression of adhesion molecules in MOVACs

To determine the effect of RKIP on the TNF-α-induced expression of ICAM-1 and VCAM-1 in MOVACs, the cells were treated with TNF-α and RKIP. The results showed that RKIP effectively upregulated the expression of RKIP (Figure 1A, 1B). The mRNA expression levels of ICAM-1 and VCAM-1 were significantly increased by TNF-α treatment. When the MOVACs were transfected with the RKIP overexpression vector, the expression levels of ICAM-1 and VCAM-1 were significantly suppressed, compared with those in the cells treated with TNF-α alone. By contrast, treatment of the MOVACs with RKIP alone had no significant effect on the expression levels of ICAM-1 or VCAM-1, compared with the control (Figure 1C, 1D).

RKIP suppresses TNF-α-induced expression of adhesion molecules in MOVACs

As the aforementioned experiments demonstrated that the overexpression of RKIP significantly inhibited the surface expression of ICAM-1 and VCAM-1 in the MOVACs, the effects of RKIP on the transcription of adhesion molecules were measured. As shown in Figure 2A–2D, the overexpression of RKIP suppressed the mRNA and protein expression levels of ICAM-1 and VCAM-1 in the TNF-α-treated MOVACs cells. Treatment with RKIP alone had no significant effect on the expression levels of ICAM-1 and VCAM-1, compared with the untreated control. These results suggested that the expression of ICAM-1 and VCAM-1 induced by TNF-α was transcriptionally modulated by RKIP.
RKIP suppresses the adhesion of THP-1 cells to TNF-α-activated MOVACs

The present study also examined the effect of RKIP on the adhesion of THP-1 cells to TNF-α-activated MOVACs. The results showed that TNF-α significantly promoted the adhesion of THP-1 to the MOVACs, and RKIP transfection markedly suppressed the adhesion of THP-1 cells to TNF-α-activated MOVACs. No significant differences were found between the MOVACs treated with RKIP alone and the untreated control (Figure 3).

RKIP inhibits TNF-α-induced NF-kB signaling in MOVACs

The NF-κB signaling pathway is important in the expression of adhesion molecules. Therefore, the effect of RKIP on the activation of NF-kappaB transcription was assessed in the present study. The results showed that luciferase activity was significantly stimulated by TNF-α and decreased by the overexpression of RKIP (Figure 4A). As the nuclear p65 protein is a critical subunit of NF-kappaB, which exerts its activity in the transcription of several genes, the protein expression of p65 NF-kappaB was also evaluated in the present study. The results showed...
RKIP and NF-kappaB signaling inhibitors reduce the expression of ICAM-1 and VCAM-1 induced by TNF-α.

Following confirmation that the overexpression of RKIP led to inhibition of the TNF-α-activated NF-kappaB signaling pathway, the present study investigated the inhibitory effect of the overexpression of RKIP on the expression levels of ICAM-1 and VCAM-1, compared with MOVACs treated with the NF-kappaB signaling inhibitor BAY11-7082. The increased expression levels of ICAM-1 and VCAM-1 induced by TNF-α were decreased following transfection with RKIP or treatment with BAY11-7082 (Figure 5A, 5B). These results indicated that the TNF-α-induced expression of ICAM-1 and VCAM-1 were regulated by the NF-kappaB pathway. RKIP inhibited the TNF-α-induced expression of VCAM-1 and ICAM-1 by inhibiting activation of the NF-kappaB pathway.

Discussion

Adhesion molecules are glycoproteins on the cell membrane surface, which mediate cell-cell and cell-ECM interactions, and are widely distributed on vascular endothelial cells and the surfaces of various leukocytes. They are involved in the development of a range of chronic diseases, including rheumatoid arthritis, tumor growth, and wound repair [21,22]. Adhesion molecules are also important in angiogenesis, inflammation, and the invasion and metastasis of tumor cells due to the mediation of cellular adhesion and migration [23]. In addition to promoting angiogenesis in simplified systems, adhesion molecules can stimulate mesenchymal cells to generate ECM proteins through the TNF-α cytokine, altering endothelial cell morphology and behavior [24]. This is beneficial to tumor cell adhesion, migration, invasion and metastasis. Currently, RKIP, as an inhibitor of key signal transduction cascades, is a promising adhesion suppressor; however, the molecular mechanisms underlying its regulation of the activity of adhesion molecules remain to be fully elucidated.

In the present study, TNF-α was used to stimulate vascular smooth muscle cells, following which the TNF-α-induced cells were transfected with an RKIP overexpression vector. The up-regulation of RKIP suppressed the TNF-α-induced increase in the expression of adhesion molecules ICAM-1 and VCAM-1 in the vascular smooth muscle cells. Vascular smooth muscle cells express ICAM-1 and VCAM-1, which are reported to be prominent in the fibrous caps of advanced atherosclerotic plaques and are also associated with disease severity [25]. Similar results were obtained in a previous study, in which the overexpression of RKIP in a cellular model used to examine the behavior of differentiated epithelial cells resulted in the loss of cell-cell contacts and increased migratory capacity of cells, whereas RKIP silencing reduced the rate of cell migration [26].

In the present study, the overexpression of RKIP prevented the adhesion of THP-1 to TNF-α-activated MOVACs. The potential mechanism may be that TNF-α induced monocyte-MOVAC interaction, increasing the local production and activity of matrix metallo proteinase (MMP)-1, which has an important pathogenic role in atherosclerotic plaque rupture [27]. A member of the zinc-dependent proteolytic enzyme family, MMP-1 can effectively degrade the ECM, which is important in tumor growth and metastasis. Therefore, RKIP reduced the TNF-α-induced expression of cellular adhesion molecules causing a decrease in monocyte-MOVAC interaction, and resulting in decreased MMP-1, a reduction in ECM degradation, and the alleviation of tumor cell migration and metastasis.

The NF-κB signaling pathway is important in the expression of adhesion molecules. As the overexpression of RKIP alters NF-κB signaling induced by TNF-α, independent of the regulatory effects of the mitogen-activated protein kinase cascade [28], the present study examined the effect of RKIP on the IκB/NF-κB pathway to explain the molecular mechanism underlying the effect of the overexpression of RKIP in reducing
the TNF-α-induced expression of adhesion molecules. The results showed that RKIP inhibited the TNF-α-induced transcriptional activation of NF-κB and nuclear protein expression of p65, a critical subunit of NF-κB. It also suppressed the phosphorylation of IκB-α to inhibit the degradation of IκB-α in TNF-α-induced MOVACs. This suggested that RKIP inhibited the TNF-α-induced nuclear translocation of NF-κB through inhibiting IκB degradation of its suppressive protein, leading to the downregulation of p65, which is a binding protein of NF-κB. A previous study also showed that RKIP inhibited TNF-α-induced NF-κB through inhibiting IKKβ, resulting in a reduction in the ability of IKK to phosphorylate or activate IκBα and p-IκBα. 

**Figure 4.** Effect of RKIP on the activation of NF-kappaB and degradation of IκB-α in TNF-α-treated MOVACs. (A) NF-kappaB activity was determined using a Dual-Luciferase reporter assay system. The protein expression of (B) p65, (C) p-IκB-α, and (D) IκB-α were detected using western blot analysis. The data presented are representative of three independent experiments performed in triplicate. Values are presented as the mean ± standard error of the mean. * p<0.05 versus control; # p<0.05 versus TNF-α-stimulated cells not transfected with RKIP. TNF-α – tumor necrosis factor-α; RKIP – Raf kinase inhibitor protein; NF-kappaB – nuclear factor-kB; IκB-α – inhibitor of NF-kappaB-α; p- – phosphorylated.
allowing NF-κB to remain sequestered with IκB [5,7,9,28]. RKIP appears to have multiple functions in the regulation of different cell signaling cascades. It is initially present on the surface of cells, although it does not have a recognized secretory signal and may only act locally. However, the recombinant overexpression of RKIP in rat fibroblast cells results in the detection of RKIP in the cell medium [29]. The mechanism through which RKIP is released from cells remains to be elucidated and requires further investigation. In terms of the present study, one possibility is that RKIP was secreted through non-classical signal secretory mechanisms, which may be through interactions with granule membrane lipids and lipid rafts.

Based on the aforementioned, the present study investigated the effect of NF-kappaB signaling on the regulation of adhesion molecules in the TNF-α-stimulated MOVACs. The overexpression of RKIP relieved cell-cell adhesion in monocytes and MOVACs through downregulation of the degradation of IκB and expression of p65. This inhibited the nuclear translocation of NF-kappaB induced by TNF-α, inhibiting the effect of NF-kappaB signaling on the regulation of adhesion molecule expression. These findings may contribute to the identification of potential therapeutic targets for inflammatory diseases, cardiovascular disease, and the migration and metastasis of malignant tumors. However, the potential involvement of other signaling pathways and the precise underlying mechanisms require further investigation.

Conclusions

The present study found that RKIP appeared to have a negative regulatory effect on cell-cell adhesion in monocytes and MOVACs. The overexpression of RKIP relieved cell-cell adhesion in monocytes and MOVACs through downregulation of the degradation of IκB and expression of p65. This inhibited the nuclear translocation of NF-kappaB induced by TNF-α, inhibiting the effect of NF-kappaB signaling on the regulation of adhesion molecule expression. These findings may contribute to the identification of potential therapeutic targets for inflammatory diseases, cardiovascular disease, and the migration and metastasis of malignant tumors. However, the potential involvement of other signaling pathways and the precise underlying mechanisms require further investigation.

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