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TNF- $\alpha$  enhances neutrophil adhesiveness: Induction of VCAM-1 via activation of Akt and CaM kinaseII and modifications of HAT and HDAC4 in human tracheal smooth muscle cells

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**Running title page:**

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**ABBREVIATIONS:** BAL, Bronchoalveolar lavage; CBP, CREB-binding protein; ChIP, Chromatin immunoprecipitation; COPD, Chronic obstructive pulmonary disease; DEPC, Dulbecco's modified Eagle's medium; EGFR, Epidermal growth factor receptor; HATs, Histone acetyltransferases; HTSMCs, Human tracheal smooth muscle cells; HBECs, Human bronchial epithelial cells; JNK, c-Jun-NH<sub>2</sub>-terminal kinase; PI3K, Phosphatidylinositol 3-kinase; RTK, Receptor tyrosine kinase; TTBS, Tween-Tris-buffer saline; CaMKII, calmodulin-dependent kinase-II; TRANCE, TNF-related activation-induced cytokine

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## ABSTRACT

Up-regulation of VCAM-1 involves adhesions between both circulating and resident leukocytes and the human tracheal smooth muscle cells (HTSMCs) during airway inflammatory reaction. We have previously demonstrated that TNF- $\alpha$ -induced VCAM-1 expression is regulated by MAPKs, NF- $\kappa$ B and p300 activation in HTSMCs. In addition to this pathway, phosphorylation of Akt and CaM kinase II has been implicated in HAT and HDAC4 activation. Here, we investigated whether these different mechanisms participating in TNF- $\alpha$ -induced VCAM-1 expression and enhanced neutrophils adhesion. TNF- $\alpha$  significantly increased HTSMCs-neutrophil adhesions, and this effect was associated with increased expression of VCAM-1 on the HTSMCs and was blocked by the selective inhibitors of Src (PP1), EGFR (AG1478), PI3K (LY294002 and wortmannin), calcium (BAPTA/AM), PI-PLC (U73122), PKC (GÖ6976, rottlerin, and Ro 31-8220), CaM (calmidazolium chloride), CaM kinase II (KT5926 and KN62), p300 (curcumin), and HDAC (trichostatin A) or transfection with siRNAs for Src, Akt, PKC $\alpha$ , PKC $\mu$  and HDAC4. At gene regulation level, RT-PCR and promoter assays revealed that expression of VCAM-1 was also attenuated by these signaling molecule inhibitors. Moreover, TNF- $\alpha$  induced Akt and CaM kinase II phosphorylation via cascades through Src/EGFR/PI3K and PLC/calcium/CaM, respectively. Finally, activation of Akt and CaM kinase II may eventually lead to the acetylation of histone residues and phosphorylation of histone de-acetylase. These findings revealed that TNF- $\alpha$  induced VCAM-1 expression via multiple signaling pathways. Blockade of these pathways may be selectively targeted to reduce neutrophil adhesion via VCAM-1 suppression and attenuation of the inflammatory responses in airway diseases.

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## Introduction

Tumor necrosis factor (TNF)- $\alpha$  initiates numerous changes of gene expression in tracheal smooth muscle cells (TSMCs) that contributes to the pathology of various diseases including airway inflammation (Yang et al., 2005; Mukhopadhyay et al., 2006; Berry et al., 2006). Recently, we have demonstrated that up-regulation of vascular cell adhesion molecule (VCAM)-1 induced by TNF- $\alpha$  enhances adhesions between both circulating and resident leukocytes and TSMCs which might trigger inflammatory reaction. Our results have demonstrated TNF- $\alpha$ -mediated signaling to activate ERK1/2, p38 and JNK1/2 and nuclear factor (NF)- $\kappa$ B pathways and reveal the critical roles of cytokines TNF- $\alpha$  and IL-1 $\beta$  in VCAM-1 expression in human TSMCs (Lee et al., 2006; Wang et al., 2005). Previous studies have also shown that TNF- $\alpha$ -mediated induction of VCAM-1 gene transcription in endothelial cells involves several transcription factors including NF- $\kappa$ B, GATA-2, IRF-1, and AP-1 (Inoue et al., 2006). In addition, enhanced activity of PI3K/Akt by ligands may control several cellular responses including cell growth, survival and migration (Kwak et al., 2003; Koyasu, 2003; Guha and Mackman, 2002). For example, in endothelial cells, TNF-related activation-induced cytokine (TRANCE)-stimulated expression of ICAM-1 and VCAM-1 is mediated by activation of PLC and PI3K-dependent PKC- $\alpha$  and PKC- $\zeta$  (Min et al., 2005). Tong *et al.* (2006) have reported that hypoxia-induced mitogenic factor induces VCAM-1 production in mouse lung tissues and epithelial cells which is dependent on activation of PI3K/Akt/NF- $\kappa$ B. Moreover, in a murine asthma model, intratracheal administration of PI3K inhibitors or AdPTEN remarkably reduces bronchial inflammation and airway hyperresponsiveness (Kwak et al., 2003; Lee et al., 2006).

Besides these specific transcription factor-mediated signaling axes, general protein acetylation has been shown to influence a broad set of cellular responses including diverse aspects of transcriptional regulation through the recruitment of two classes of enzymes, in particular, histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Roth et al., 2001). In asthma patients, there is a marked increase in HAT and a small reduction in HDAC activities compared with those of normal airways (Barnes et al., 2005). The p300/CBP proteins are endowed with HAT activity, which transfers an acetyl group to the core histones of a lysine residue, and the acetylation level of chromatin has been established to be a key mechanism in regulating inflammatory gene transcription (Chan and La Thangue, 2001), such as VCAM-1 (Lee et al., 2006), COX-2 (Nie et al., 2003) and MMP-9 (Ma et al., 2005). Many studies have reported the regulation of p300 or CBP by protein kinases, including PKCs (Yuan and Gambee, 2000), CaMKIV (Impey et al., 2002), and p38 (Poizat et al., 2005). In addition, p300/CBP consisting of an RXXRXXpS/T consensus sequence in the C-terminal, where X is any amino acid, R is arginine, and pS/T is phosphorylated serine or threonine,

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preferred by Akt has been involved in TNF- $\alpha$ -induced ICAM-1 expression (Huang and Chen, 2005). Our current findings also suggest that upon TNF- $\alpha$  treatment, p300 could be recruited to the VCAM-1 promoter, which may be regulated by these kinases.

Conversely, gene repression is mediated via HDACs, which catalyze deacetylation by cleaving acetyl groups, resulting in tightening of nucleosomal integrity and suppression of transcription (de Ruijter et al., 2003). There are multiple mammalian HDACs, which fall into 3 classes on the basis of structural and biochemical characteristics. Class I deacetylases (HDACs 1, 2, 3 and 8); Class II deacetylases include HDACs 4, 5, 6, 7, 9 and 10; the third class of HDACs is the conserved nicotinamide adenine dinucleotide-dependent Sir2 family of deacetylases (Lin et al., 2006). Many transcription factors have been shown to associate with HDAC to regulate gene transcription. For instance, corticosteroids appear to suppress inflammation in asthma by switching off these inflammatory genes by targeting HDAC2. HDAC2 is able to deacetylate acetylated NF- $\kappa$ B and promotes its association with the inhibitor I $\kappa$ B- $\alpha$  within the nucleus leading to export into the cytoplasm and thus terminates the activity of NF- $\kappa$ B (Barnes et al., 2005). Most interestingly, class II HDACs possess the capability of activating nucleo-cytoplasmic shuttling, which are suggested to be regulated by CaMK-dependent phosphorylation. For instance, phosphorylation of HDAC4 by CaMKII promotes nuclear export and prevents nuclear import of HDAC4, with consequent de-repression of HDAC4 targeting genes and results in hypertrophic growth (Backs et al., 2006; Little et al., 2007; Liu et al., 2005). In analogy, another study has proposed a novel mechanism which CaMKIV mediates the phosphorylation of HDAC4, thus allowing IL-5 gene transcriptional activation (Han et al., 2006). These findings reveal a central role for HDAC4 in CaMK signaling pathways and have implications in the control of gene expression by calcium signaling in a variety of cell types.

In this presentation, we investigated the different signal intermediates coupled TNF- $\alpha$  to distinct subsets of target genes such as VCAM-1 in HTSMCs. We showed that TNF- $\alpha$ -mediated VCAM-1 induction was indeed governed by transcript-specific signaling pathways, involving the Src/EGFR/PI3K and PLC/Ca<sup>2+</sup>/CaMK. Both pathways activate Akt and CaM kinase II may eventually lead to the acetylation of histone residues and phosphorylation of histone de-acetylase. Blockade of these pathways may selectively target to reduce neutrophil adhesion via VCAM-1 suppression and attenuation of the inflammatory response in airway diseases.

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## Materials and Methods

**Reagents.** DMEM/F-12 medium, FBS, and TRIZOL were purchased from Invitrogens (Carlsbad, CA, USA). [ $\gamma$ - $^{32}$ P]ATP (6000 Ci/mmol), Hybond C membrane, enhanced chemiluminescence (ECL) Western blotting detection system and Hyperfilms were from Amersham Biosciences (Buckinghamshire, England). Polyclonal antibodies VCAM-1, HDAC4 and NF- $\kappa$ B (p65) Ab were from Santa Cruz (Santa Cruz, CA, USA). Anti-GAPDH antibody was from Biogenesis (Boumemouth, UK). Specific CaM Kinase II Assay kit was from Upstate (Lake Placid, NY, USA). CaM kinase II, phospho CaM kinase II (Thr<sup>286</sup>) and phospho Akt Ab kits were from Cell Signaling (Beverly, MA, USA). PP1, AG1478, LY294002, wortmannin, curcumin, U73122, BAPTA, KT5926, KN62, calmidazolium chloride, trichostatin A (TSA), GÖ6976, Ro 31-8220, rottlerin and MG132 were from Biomol (Plymouth Meeting, PA, USA). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL, USA). Enzymes and other chemicals were from Sigma (St. Louis, MO, USA).

**Cell Culture.** HTSMCs were purchased from ScienCell Research Lab (San Diego, CA) and cultured as described previously (Lee et al., 2004). When the cultures reached confluence, cells were treated with 0.05% (w/v) trypsin/0.53 mM EDTA for 5 min at 37°C. The cell suspension was diluted with DMEM/F-12 containing 10% FBS to a concentration of  $2 \times 10^5$  cells/ml. The cell suspension was plated onto (1 ml/well) 12-well culture plates and (10 ml/dish) 10-cm culture dishes for the measurement of protein expression and mRNA accumulation, respectively. Experiments were performed with cells from passages 3 to 8.

**Preparation of Cell Extracts and Western Blot Analysis.** Growth-arrested HTSMCs were incubated with TNF- $\alpha$  at 37°C for the indicated time. The cells were washed with ice-cold PBS, scraped, collected, and centrifuged at 45,000  $\times g$  for 1 h at 4°C to yield the whole cell extract, as previously described (Lee et al., 2004). Samples were denatured, subjected to SDS-PAGE using a 10% running gel, and transferred to nitrocellulose membrane. Membranes were incubated overnight at 4°C with an anti-VCAM-1, anti-HDAC4, anti-p65, anti-p50 or anti-GAPDH antibody used at a dilution of 1:2000 in 5% (w/v) BSA in TTBS [(50 mM Tris-HCl, 150 mM NaCl, 0.05% (w/v) Tween 20, pH 7.4)]. Membranes were incubated with a 1:2000 dilution of anti-goat or anti-mouse horseradish peroxidase antibody for 1 h. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

**Transfection of siRNAs for Src, Akt, PKC $\alpha$  and PKC $\mu$ , p300 and HDAC4.** Smartpool RNA duplexes corresponding to Src kinase, Akt kinase, PKC $\alpha$  and PKC $\mu$  kinase, p300, HDAC4 and scrambled control #2 siRNA were purchased from Dharmacon Research Inc (Lafayette, CO, USA). HTSMCs (P4 or P5) were cultured onto 12-well plates. At 70-80% confluence, transient transfection of siRNA was carried out using Metafectene Transfection

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Reagent (Biontexas). Briefly, siRNA (100 nM) was formulated with DNA-Metafectene Transfection according to the manufacturer's instruction. The transfection complex was diluted into 900  $\mu$ l of DMEM/F12 medium and added directly to the cells. The medium was replaced with complete basal essential growth medium after 3 h. Cells were analyzed at 72 h after transfection by Western blotting.

**Total RNA Extraction and RT-PCR Analysis.** Total RNA was isolated from HTSMCs treated with TNF- $\alpha$  in 10-cm culture dishes with Trizol. RNA concentration was spectrophotometrically determined at 260 nm. First strand cDNA synthesis was performed with 2  $\mu$ g of total RNA using random hexamers as primers in a final volume of 20  $\mu$ l (5  $\mu$ g/ $\mu$ l random hexamers, 1 mM dNTPs, 2 units/ $\mu$ l RNasin, and 10 units/ $\mu$ l moloney murine leukemia virus reverse transcriptase). The reaction was carried out at 37°C for 60 min. cDNAs encoding  $\beta$ -actin or VCAM-1 was amplified from 3-5  $\mu$ l of the cDNA reaction mixture using specific gene primers. Oligonucleotide primers for  $\beta$ -actin and VCAM-1 were as follow:

$\beta$ -actin:

5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'(sense),

5'-CTAGAAGCATTGCGGTGGACGATG-3' (anti-sense);

VCAM-1:

5'-GGAACCTTGCAGCTTACAGTGACAGAGCTCCC-3'(sense),

5'-CAAGTCTACATATCACCCAAG-3' (anti-sense)

The amplification profile includes 1 cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C and extension at 72°C for 1 min then 1 cycle of final extension at 72°C for 5 min. The expression of  $\beta$ -actin was used as an internal control.

**Nuclear Extract.** HTSMCs were seeded in a 10-cm dish. After they reached 90% confluence, the cells were starved for 24 h in serum-free DMEM/F-12 medium. After stimulation with 30 ng/ml TNF- $\alpha$ , the cells were washed, scraped, and centrifuged to prepare cytosolic and nuclear fractions, as previously described (Wang et al., 2005). The nuclear export of HDAC4 and translocation of p65 or p50 was identified by Western blot analysis using HDAC4, p65 and p50 antibody.

**Measurement of VCAM-1 Luciferase Activity.** For construction of the VCAM-1-luc plasmid, human VCAM-1 promoter, a region spanning -1716 to -119 bp (kindly provided by Dr. W.C. Aird, Department of Molecular Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA) was cloned into pGL3-basic vector. VCAM-1-luc plasmid was transiently transfected into HTSMCs using Genejammer Transfection Reagent (Stratagene, La Jolla, CA, USA). Briefly, plasmids (1.8  $\mu$ g) and  $\beta$ -gal (0.2  $\mu$ g) were formulated with Genejammer transfection according to the manufacturer's instruction. The transfection complex was

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diluted into 900  $\mu$ l of DMEM/F-12 medium and added directly to the cells. The medium was replaced with complete basal essential growth medium after 5 h. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system. Firefly luciferase activities were standardized for  $\beta$ -galactosidase activity.

**Chromatin Immunoprecipitation Assay.** To detect the *in vivo* association of nuclear proteins with human VCAM-1 promoter, chromatin immunoprecipitation (ChIP) analysis was conducted as described previously (Nie et al., 2003) with some modifications. HTSMCs in 100-mm dishes were grown to confluence and serum starved for 24 h. After treatment with TNF- $\alpha$ , protein-DNA complexes were fixed using 1% formaldehyde in PBS. The fixed cells were washed and lysed in SDS-lysis buffer (1% SDS, 5 mM EDTA, 1 mM PMSF, 50 mM Tris-HCl, pH 8.1) and sonicated on ice until the DNA size became 200~1000 base pairs. The samples were centrifuged, and the soluble chromatin was pre-cleared by incubation with sheared salmon sperm DNA-protein agarose A slurry (Upstate) for 30 min at 4°C with rotation. After centrifugation at 800 rpm for 1 min, one portion of the pre-cleared supernatant was used as DNA input control, and the remains were subdivided into aliquots and then incubated with a non-immune rabbit immunoglobulin G (IgG; Upstate), anti-p300 Ab (sc-585; Santa Cruz), anti-acetylated histone H4 Ab (06-866; Upstate), or anti-HDAC4 Ab (sc-11418, Santa Cruz), respectively, overnight at 4°C. The immuno-precipitated complexes of Ab-protein-DNA were collected using protein A agarose, and washed successively with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high-salt buffer (same as the low-salt buffer but with 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and Tris-EDTA (pH 8.0), and then eluted with elution buffer (1% SDS, 100 mM NaHCO<sub>3</sub>). The cross-linking of protein-DNA complexes was reversed by incubation with 5 M NaCl at 65°C for 4 h, and DNA was digested with 10  $\mu$ g/ml of proteinase K (Sigma) for 1 h at 45°C. The DNA was then extracted with phenol-chloroform, and the purified DNA pellet was precipitated with isopropanol. The DNA pellet was re-suspended in H<sub>2</sub>O and subjected to PCR amplification with the forward primer 5'-AAATCAATTCACATGGCATA-3' and the reverse primer 5'-AAGGGTCTTGTTGCAGAGG-3', which were specifically designed from the VCAM-1 promoter region (-403 to -30). PCR products were analyzed on ethidium bromide-stained agarose gels.

**CaM Kinase Activity.** HTSMCs in 10-cm dishes were grown to confluence and serum starved for 24 h. Cells were treated with 30 ng/ml TNF- $\alpha$  for the indicated times or pretreated with 10  $\mu$ M KN62 for 1 h and then incubated with TNF- $\alpha$  for 10 min. Whole cell lysates

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were immunoprecipitated with an anti-CaM kinase II Ab overnight at 4°C. The immunoprecipitated proteins were diluted with kinase buffer and analyzed by CaM Kinase II Assay Kit. Briefly, 10 µl of sample was added to an eppendorff vial containing 10 µl of ADBII, 10 µl of CaM kinase II substrated cocktail, 10 µl of PKA and PKC inhibitor cocktail, 10 µl of [ $\gamma$ -<sup>32</sup>P]ATP (10 mCi/ml) and then incubated for 10 min at 30°C. After incubation, 25 µl of reaction mixture was spotted on numbered P81 paper which was rinsed sequentially using 0.75% phosphoric acid for six times and once with acetone. The P81 paper was then transferred to a scintillation vial and added scintillation cocktail. The radioactivity was counted using a scintillation counter (Beckman LS5000TA, Fullerton, CA, USA). The specific activity of CaM kinase II was calculated according to the directions of manufacturer.

**Immunofluorescence Staining.** HTSMCs were plated on six-well culture plates with coverslips. Cells were treated with 30 ng/ml TNF- $\alpha$  in the absence or presence of 10 µM KN62 and washed twice with ice-cold PBS. Immunofluorescence staining was performed as previously described (Lee et al., 2006). The staining was performed by incubating with 10% normal goat serum in PBS for 30 min followed by incubating with anti-HDAC4 Ab (1:100 dilution) for 1 h in PBS with 1% BSA, washing three times with PBS, incubating for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ab (1:100 dilution) in PBS with 1% BSA, washing three times with PBS, and finally mounting with aqueous mounting medium. The images observed under a fluorescence microscope (Nikon, Optiphot 2/EFD2).

**Neutrophil Adhesion Assay.** Peripheral blood PMNs were isolated from whole venous blood by dextran sedimentation followed by density separation over Ficoll-Hypaque and hypotonic lysis. The PMN were then resuspended in Tyrode-HEPES buffer (128 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 10 mM HEPES; pH 7.4) and adjusted to 1X10<sup>7</sup> cells/ml. PMN were used within 4 h of purification.

Adhesion of neutrophils to HTSMCs was measured as described previously (Chen et al., 2001). Briefly, neutrophils were labeled with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and added to an HTSMCs monolayer. Non-adherent cells were removed by gentle washing with PBS, and the number of adherent cells was determined by measuring the fluorescence intensity using a CytoFlour 2300 (Millipore, Bedford, MA, USA).

**Statistical Analysis.** Data were analyzed with GraphPad Prism Program (GraphPad, San Diego, CA, USA) and expressed as the mean  $\pm$  S.E.M. and analyzed with a two-tailed Student's t-test at a P<0.05 level of significance.

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## Results

**TNF- $\alpha$ -Enhanced Neutrophils Adhesion Is Inhibited by PP1, AG1478, LY294002 and VCAM-1 Antibody.** Recently, we have reported that TNF- $\alpha$ -stimulated VCAM-1 expression in HTSMCs is dependent on MAPKs and NF- $\kappa$ B (Lee et al., 2006). To further investigate whether an alternative pathway of Src/EGFR/PI3K/Akt was required for TNF- $\alpha$ -induced VCAM-1 expression, HTSMCs were pretreated with specific inhibitors or transfected with siRNAs. Transfection of HTSMCs with Akt or Src siRNAs for 72 h down-regulated expression of respective proteins by ~90% as compared with control siRNA-transfected cells. As shown in Figs. 1A and B, TNF- $\alpha$ -induced VCAM-1 protein and mRNA expression was significantly inhibited by pretreatment with 10  $\mu$ M PP1, 10  $\mu$ M AG1478, 10  $\mu$ M LY294002 and 10  $\mu$ M wortmannin as well as transfection with siRNA for Src and Akt. Treatment with vesicle alone had no effect on the basal level of VCAM-1 (data not shown). These findings suggested that TNF- $\alpha$ -induced VCAM-1 expression may be mediated through Src, EGFR, and PI3K/Akt cascades. Moreover, pretreatment of HTSMCs with inhibitors of Src (PP1), EGFR (AG1478) and P13K (LY294002) for 1 h or transfection with siRNA of Src and Akt prior to exposure to TNF- $\alpha$  for 30 min caused an attenuation of Akt phosphorylation (Fig. 1C). However, TNF- $\alpha$  stimulated phosphorylation of Akt was not inhibited by calmodulin inhibitor (calmidazolium chloride) and CaMKII inhibitor (KT5926) (Data not shown). To further determine the functional consequence of selective signaling inhibitors, we performed cell adhesion assays using human neutrophils adhered to HTSMCs pretreated with TNF- $\alpha$  for 24 h. The number of adherent neutrophils dramatically increased which was attenuated by pretreatment with PP1, AG1478 and LY294002, respectively (Fig. 1D). In particular, the adhesion activity was also blocked by an anti-VCAM-1 antibody. These results indicated that TNF- $\alpha$ -induced VCAM-1 expression was mediated through Src, EGFR, and PI3K/Akt in HTSMCs and enhanced neutrophil-HTSMC interactions.

**The Recruitment of p300 to VCAM-1 Promoter by Akt Phosphorylation Is Required for TNF- $\alpha$ -Induced VCAM-1 Expression.** p300, one of histone acetyl-transferase, belongs to a large class of transcription co-activators, which serves as adaptors for transcriptional activation of diverse genes. The p300 has been shown to up-regulate VCAM-1 transcription (Lee et al., 2006), suggesting an important role of p300 in bridging the multiple DNA-bound transactivators with transcription factors to initiate VCAM-1 transcription. TNF- $\alpha$ -induced neutrophil adhesion (Fig. 2A) and VCAM-1 mRNA expression (Fig. 2B) were inhibited by pretreatment with curcumin (a novel specific inhibitor of p300) and MG132 (a proteasome inhibitor).

It has been reported that p300 act as protein bridges, thereby connecting different

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transcriptional factors or activators via protein-protein interactions to the transcriptional machinery, such as TFIIB, RNA polymerase II complex, AP-1, and NF- $\kappa$ B (Deng et al, 2003; Chan and La Thangue, 2001). In the present study, to further confirm the involvement of p300 in TNF- $\alpha$ -induced VCAM-1 expression, HTSMCs were transfected with p300 siRNA. As shown in Fig. 2C, transfection of HTSMCs with p300 siRNA down-regulated p300 protein and attenuated TNF- $\alpha$ -induced VCAM-1 expression determined by Western blotting against an anti-p300 or VCAM-1 antibody, respectively. Moreover, we presented evidence that NF- $\kappa$ B interacted with p300 and histone H4 in vitro, immunoprecipitation of nuclear lysates with a p300 Ab, followed by immunoblot analysis using anti-p65, anti-p50 and anti-phospho-Akt Ab. As shown in Fig. 2D, p300 and histone H4 formed a complex with NF- $\kappa$ B after TNF- $\alpha$  stimulation, which was blocked by LY294002, helenalin (a NF- $\kappa$ B inhibitor, Lyss et al., 1998) and curcumin. Furthermore, the roles of these transcription factors and the VCAM-1 promoter regulatory elements in TNF- $\alpha$ -induced gene transcription, in vivo association of p300 with the VCAM-1 promoter was evaluated by a ChIP assay. To determine whether p300 could associate with the VCAM-1 promoter, PCR amplifications were conducted on an equal amount of immunoprecipitated DNA, followed by 45 cycles of PCR with the specific primer pairs encompassing position -403 to -30 regions of human VCAM-1 promoter, which contains NF- $\kappa$ B binding sites. After TNF- $\alpha$  treatment, an enrichment of p300- and histone H4-associated VCAM-1 promoter DNA appeared within 30 min and sustained up to 2 h, as compared with the non-immune IgG immunoprecipitated control. Association of p300 and histone H4 with the VCAM-1 promoter was blocked by pretreatment with LY294002 (Fig. 2E) or helenalin (Fig. 2F). These data indicated that NF- $\kappa$ B, p300, and histone H4 were involved in TNF- $\alpha$ -induced VCAM-1 transcription and regulated by a PI3K/Akt- and NF- $\kappa$ B-dependent pathway in HTSMCs.

**CaM and CaMKII Regulate TNF- $\alpha$ -Induced VCAM-1 Expression and Neutrophils Adhesion.** Previously, Min et al. (2005) have demonstrated that TRANCE-induced the expression of ICAM-1 and VCAM-1 in endothelial cells is regulated by PLC and calcium-dependent pathway. To determine whether PLC and Ca<sup>2+</sup> mediated TNF- $\alpha$ -induced VCAM-1 expression, HTSMCs were treated with 30 ng/ml TNF- $\alpha$  in the presence of various signaling inhibitors and revealed expression of VCAM-1 by Western blotting and semi-quantitative RT-PCR. As shown in Figs. 3A and B, pretreatment with inhibitors of PLC (U73122), Ca<sup>2+</sup> chelator (BAPTA-AM), calmodulin (calmidazolium chloride), and CaMKII (KT5926), suppressed TNF- $\alpha$ -induced VCAM-1 expression at both protein and mRNA levels. In addition, KN-62 (a CaMKII inhibitor) was used to confirm the involvement of CaMKII in TNF- $\alpha$ -induced responses. As shown in Fig. 3C, pretreatment with KN62 inhibited TNF- $\alpha$ -induced VCAM-1 expression in a concentration-dependent manner.

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It has been demonstrated that several agonists potently increase  $\text{Ca}^{2+}$  and the activity of the  $\text{Ca}^{2+}$ /CaMKII (Marganski et al., 2005; Soderling et al., 2001). The involvement of CaMKII phosphorylation in TNF- $\alpha$ -induced VCAM-1 expression was detected using U73122, BAPTA/AM, calmidazolium chloride and KT5926, analyzed by Western blotting with an anti-phospho-CaMKII antibody. TNF- $\alpha$ -enhanced phosphorylation of CaMKII was inhibited by pretreatment with U73122, BAPTA-AM, calmidazolium chloride and KT5926 (Fig. 3D), but not by PP1 or LY294002 (data not shown). Furthermore, we have also determined the CaM kinase activity using a CaM Kinase II Assay Kit according to the directions of manufacturer (Upstate). As shown in Fig. 3E, TNF- $\alpha$ -induced a significant increase in CaMKII activity in a time-dependent manner (3.3 fold over the basal level within 10 min), which was inhibited by pretreatment with KN62. To further confirm whether the inhibitors for CaM and CaMKII also attenuated the adherent function via attenuation of VCAM-1 expression, we pretreated HTSMCs with calmidazolium chloride and KT5926 for 1 h and then incubated with TNF- $\alpha$  for 24 h prior to addition of neutrophils. As shown in Fig. 3F, TNF- $\alpha$ -enhanced neutrophil adhesion to HTSMCs was reduced by pretreatment with calmidazolium chloride and KT5926, respectively. Taken together, these results suggested that selective inhibition of PLC/ $\text{Ca}^{2+}$ /CaM/CaMKII signaling led to a significant attenuation in TNF- $\alpha$ -induced VCAM-1 expression and neutrophil adhesion to cultured HTSMCs.

**Nuclear Export of HDAC4 Is Induced by CaMKII.** Recent study has demonstrated that TSA (a class I and II HDAC inhibitor) and other HDAC inhibitors block TNF- $\alpha$ -induced VCAM-1 expression and VCAM-1-dependent leukocyte adhesion under in vitro and in vivo conditions (Inoue et al., 2006). In addition, as a member of the class II HDACs, HDAC4 possesses the capability of nucleo-cytoplasm shuttling. The subcellular localization of HDAC4 is suggested to be regulated by CaMK. Upon phosphorylation, HDAC4 binds to a partner protein 14-3-3 which leads to an efficient nuclear export (Backs et al., 2006; Han et al., 2006). To further confirm if TNF- $\alpha$  induced nuclear export of HDAC4 through CaMKII, cells were stimulated by 30 ng/ml TNF- $\alpha$  for the indicated times. The cytosolic and nuclear fractions were then used for determination of HDAC4 translocation. As shown in Fig. 4A, TNF- $\alpha$ -induced an exportation of HDAC4 out of nucleus into cytoplasm in a time-dependent manner, with a maximal effect within 60 min, sustained up to 4 h, and then declined to the basal level. Such effect can be reversed by pretreatment with calmidazolium chloride and KT5926 (Fig. 4B). In addition, the involvement of CaMK in HDAC4 translocation was assessed by an immunofluorescence staining. As shown in Fig. 4C, TNF- $\alpha$ -induced an exportation of HDAC4 out of nucleus in a time-dependent manner, consistent with the data presented in Fig. 4A. Pretreatment with KN62 (a CaMKII inhibitor) also caused an inhibition of HDAC4 exportation.

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Furthermore, in ChIP assays aiming to test the influence of CaMKII phosphorylation on the recruitment of HDAC4 to the VCAM-1 promoter, we found that phosphorylation of CaMKII decreased the binding of HDAC4 in VCAM-1 promoter region. Inhibition of CaMKII by KT5926 reduced the dissociation of HDAC4 from the VCAM-1 promoter (Fig. 4D). To further ensure the involvement of HDAC4 in TNF- $\alpha$ -mediated VCAM-1 expression, transfection of HTSMCs with HDAC4 siRNAs for 72 h down-regulated total respective protein expression by ~60% as compared with control siRNA-transfected cells (Fig. 4E). In addition, transfection with siRNAs for HDAC4 or pretreatment with TSA also decreased both VCAM-1 expression and neutrophils adhesion (Figs. 4E and F). Taken together, these findings suggested that the effect of TNF- $\alpha$  on VCAM-1 expression was mediated through the CaMKII/HDAC signaling pathway.

**Involvement of PKC Isoforms in VCAM-1 Expression Induced by TNF- $\alpha$ .** As described in previous study, activation of PKC isoforms may be mediated through a Ca<sup>2+</sup>-dependent signaling. Hence, TNF- $\alpha$ -activated PKC isoforms linked to the expression of VCAM-1 was investigated in HTSMCs. Cells were incubated with 150 pg/ml TNF- $\alpha$  in the presence of PKC inhibitors, TNF- $\alpha$ -induced VCAM-1 protein expression was revealed by Western blotting. As shown in Fig. 5A, pretreatment of HTSMCs with Gö6976 (a Ca<sup>2+</sup>-dependent PKC inhibitor), rottlerin (a selective of PKC- $\delta$  inhibitor) and Ro318220 (a non-selective PKC inhibitor), significantly attenuated TNF- $\alpha$ -induced VCAM-1 expression, respectively. Moreover, it has been well established that activation of PKC could be translocated to the cell membrane. To further confirm direct PKC activation upon TNF- $\alpha$  treatment, the cytosolic and membrane fractions were then used to determine the amount of PKC isoforms using Western blotting analysis. As shown in Fig. 5B, treatment with TNF- $\alpha$  induced the translocation of PKC $\alpha$ ,  $\mu$  and  $\iota$  from the cytosol to the membrane fractions which indicated the activation of these isoforms. Long-term treatment with PMA caused a down-regulation of PKC served as negative controls. Furthermore, transfection with dominant negative mutants of PKC $\alpha$  and PKC $\iota$  significantly blocked both VCAM-1 protein and mRNA expression (Figs. 5C and D). The involvement of these PKC isoforms in TNF- $\alpha$ -induced responses was ensured by transfection with siRNAs for PKC genes including PKC $\alpha$  and PKC $\mu$ . Transfection of HTSMCs with PKC $\alpha$  and PKC $\mu$  siRNAs for 72 h down-regulated total proteins expression by ~80% as compared with control siRNA-transfected cells and thus attenuated the TNF- $\alpha$ -induced VCAM-1 expression (Fig.5E). These results indicated that TNF- $\alpha$ -induced VCAM-1 expression was mediated through activation of PKC $\alpha$ ,  $\mu$ , and  $\iota$  isoforms in HTSMCs.

**TNF- $\alpha$ -Induced VCAM-1 Promoter Activity.** This regulation of VCAM-1 gene transcription through Src/EGFR/PI3K/Akt and PLC/Ca<sup>2+</sup>/camodulin/CaMKII pathways

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induced by TNF- $\alpha$  was further confirmed by gene luciferase activity assay. HTSMCs were transfected with VCAM-1 luciferase reporter gene and then stimulated with TNF- $\alpha$  (30 ng/ml) for the indicated time. Data in Figure 6A showed that TNF- $\alpha$ -stimulated VCAM-1-luciferase activity reached a maximal response within 60 min and sustained up to 4 h. Moreover, TNF- $\alpha$ -induced VCAM-1 promoter activation was inhibited by selective inhibitors including AG1478, LY294002, U73122, BAPTA/AM, calmidazolium chloride and KT5926 for EGFR, PI3K, PLC, Ca<sup>2+</sup>, calmodulin and CaMKII, respectively (Fig. 6B). These results confirmed that TNF- $\alpha$ -stimulated VCAM-1 luciferase gene activity involved at a transcription level mediated through activation of Src/EGFR/PI3K/Akt and PLC/Ca<sup>2+</sup>/calmodulin/CaMKII pathways in HTSMCs.

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## Discussion

Many of the common airway diseases, including asthma and COPD, involve inflammation, with the coordinate expression of multiple inflammatory genes in the airway tissues. These inflammatory genes encode for the expression of cytokines, chemokines, enzymes and adhesion molecules (Barnes et al., 2005; Yang et al., 2005). Chromatin remodeling initiated by reversible acetylation of core histones by HAT and HDACs is considered as a key element in the dynamic regulation of all gene expressions (Roth et al., 2001). Alterations in balance between histone acetylation and deacetylation could modulate cellular function including cell growth, differentiation, cell death, cell–cell and cell–matrix interactions, and inflammatory responses. In fact, there is growing evidence in the physiological and pathological relevance of HAT and HDAC in the respiratory system, HDAC activity is decreased in patients with asthma, whereas HAT activity is increased in biopsy specimens (Ito et al., 2005). Thus, HDAC and HAT can be therapeutic targets for airway inflammatory diseases.

The inflammatory responses may be mediated by complex interactions between both circulating polymorphonuclear cells (PMNs) and endothelium. Others and our recent studies have been well documented that up-regulation of adhesive molecules on the cell surface of airway or lung cells plays a critical role in the inflammation action (Lee et al., 2006; Wang et al., 2005; Huang et al., 2003; Lee et al., 2003). In the present study, we have demonstrated a novel regulatory action of TNF- $\alpha$  and its underlying signaling mechanisms between HAT and HDAC associated with VCAM-1 gene expression in HTSMCs. At first, we showed that TNF- $\alpha$  enhanced HTSMC-neutrophil interaction, correlated with expression of the VCAM-1 in HTSMCs. In addition, we also demonstrated a critical role of the inflammatory transcription factor NF- $\kappa$ B and p300 (a histone acetyltransferase) in the TNF- $\alpha$ -induced transcription of VCAM-1. Furthermore, our results clearly demonstrated that Akt played an important role in mediating the TNF- $\alpha$ -dependent production of VCAM-1 in HTSMCs through the downstream activation of p300, but not NF- $\kappa$ B activation. Induction of VCAM-1 and phosphorylation of Akt in response to TNF- $\alpha$  were significantly attenuated in parallel by the inhibition of phosphorylation of Src, EGFR, and PI3K by their respective inhibitors of PP1, AG1478 and LY294002. Moreover, we employed transfection of siRNAs for Src and Akt specifically down-regulated the expression of either Src or Akt protein. Knockdown expression of these proteins also attenuated TNF- $\alpha$ -induced Akt activation and VCAM-1 expression in HTSMCs (Fig. 1). These results suggested that TNF- $\alpha$  stimulated p300 associated with VCAM-1 promoter (containing NF- $\kappa$ B binding sites) via the Src/EGFR/PI3K/Akt dependent signaling cascades triggered upon receptor engagement.

Under normal condition, HAT transfers an acetyl group to the core histones of a lysine

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residue, and the acetylation level of chromatin has been established to be a key mechanism in de-repress gene transcription. Conversely, gene repression is mediated via HDACs. Inoue *et al.* (2006) have demonstrated that TNF- $\alpha$ -induced VCAM-1 expression and VCAM-1-dependent endothelial-leukocyte adhesion were inhibited by pretreatment with TSA (a class I and II HDAC inhibitor) and transfection with siRNAs for HDAC3. In contrast, recent study reveals that TSA and NaBu (HDAC inhibitors) enhance the IL-5 promoter activity. Induction of IL-5 can be repressed by transfection with wild-type plasmid of the HDACs (Han *et al.*, 2006). Moreover, HDAC, which removes the acetyl groups from hyperacetylated histones, counteracts the effects of HAT and returns histone to its basal state, finally leads to suppression of gene transcription. Moreover, aberrant regulation of HDACs plays an important role in human carcinogenesis. It has been shown that TSA inhibits HDAC and has potent anti-tumor activity in many human cancers (Huang, 2006; Lin *et al.*, 2006). In contrast, TSA increases histone H3 acetylation of VCAM-1 and ICAM-1 promoters in lung endothelial cells (Rose *et al.*, 2005). However, in our study, we provided the evidence of HDAC inhibitors to exert as a negative regulator in VCAM-1 expression. Pretreatment with TSA or transfection with siRNA mediated knockdown of HDAC4 in HTSMCs and resulted in a significant reduction in TNF- $\alpha$ -enhanced neutrophil adhesion and VCAM-1 expression (Figs. 4E and F). These differences may be due to cell-specific or different experimental conditions.

In addition, chromatin remodeling induced by TNF- $\alpha$ , other transcription factor(s) or co-factor(s) may also interact with the VCAM-1 promoter to modulate its expression. Most interestingly, Simian virus 40 promoter factor 1 (Sp1) is one of the first transcription factors purified and cloned from mammalian cells (Dyran and Tjian, 1983; Kadonaga *et al.*, 1987), which can recognize and bind to VCAM-1 promoter, thus regulate the transcription of VCAM-1 genes. Previous study has demonstrated that cytokine-induced enhancer of the VCAM-1 gene requires constitutively bound Sp1 and induces heterodimeric NF- $\kappa$ B for maximal promoter activity (Neish *et al.*, 1995). Recent study has shown that HDAC1 is recruited by Sp1 to the promoter of Sp1-regulated genes, followed by deacetylation of Sp1 upon PMA treatment. p300 is then recruited to the gene promoter through the interaction with deacetylated Sp1 to acetylated histone H3, leading to the enhancement of the expression of 12(S)-lipoxygenase (Hung *et al.*, 2006). Therefore, understanding the molecular mechanisms by which HDAC4 and Sp1 promote VCAM-1 expression should be of considerable interesting.

On the other hands, it has been known that class II HDAC is activated by nucleo-cytoplasmic shuttling through CaMK-dependent phosphorylation (Little *et al.*, 2007; Backs *et al.*, 2006; Sato *et al.*, 2007; Huang and Chen, 2005). To further verify the nuclear

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exportation of HDAC4 induced by TNF- $\alpha$ , the ChIP assay and cytosolic/nuclear fractions were used for this purpose. Our data revealed that TNF- $\alpha$  stimulation caused HDAC4 exported from the nucleus to the cytoplasm; reversed by adding calmidazolium chloride (an inhibitor of calmodulin) and KT5926 (an inhibitor of CaMKII). Taken together, these data strongly suggested that CaMKII-dependent HDAC4 activation may play important roles in transcriptional regulation of the VCAM-1 gene, consistent with HDAC4 as a specific downstream substrate of CaMKII $\delta$ B in cardiac cells (Little et al., 2007). These results suggested that TNF- $\alpha$ -induced VCAM-1 expression was, at least in part, mediated through activation of CaMKII. But role of CaMKII isoforms in TNF- $\alpha$ -induced responses needs to be investigated in HTSMCs.

It was worth noting that the inhibitors of Src, EGFR, PI3K and PI-PLC, seemed to be able to completely inhibit TNF- $\alpha$ -induced VCAM-1 expression and neutrophil adhesion when used alone, suggesting that these pathways were interconnected. These protein kinases may converge at a step downstream to activate transcription factors such as NF- $\kappa$ B and p300 binding to VCAM-1 promoter. This interaction between p300 and NF- $\kappa$ B may be required for induction of VCAM-1 by TNF- $\alpha$ . Therefore, inhibition of one of these components was able to interrupt the signaling transduction associated with VCAM-1 expression. However, the complicated regulatory mechanisms underlying TNF- $\alpha$ -induced VCAM-1 expression need to be investigated in the future.

Next, Min *et al.* (2005) have shown that PLC, PI3K, and PKC are crucial downstream signals of TRAFs leading to expression of adhesion molecules. Our previous studies have also reported that activation of PKC $\delta$  is required for induction of COX-2 and cPLA<sub>2</sub> in rat brain astrocytes (Hsieh et al., 2005, 2007). Furthermore, PKC-stimulated phosphorylation of c-Src may eventually result in NF- $\kappa$ B activation and ICAM-1 expression (Huang et al., 2003). Herein, three PKC isoforms, namely PKC- $\alpha$ , PKC- $\mu$ , and PKC- $\iota$ , have been activated in response to TNF- $\alpha$  in HTSMCs. Moreover, TNF- $\alpha$ -induced expression of VCAM-1 was significantly attenuated by pretreatment with PKC inhibitors or transfection with dominant negative mutants of PKC $\alpha$  and PKC $\mu$ .

In summary, the overall pathway by which TNF- $\alpha$  induces VCAM-1 expression was illustrated in Fig. 7. TNF- $\alpha$  is an airway inflammatory mediator that increases expression of inflammatory molecule VCAM-1 via sequential activation of PKCs, Src/EGFR/PI3K/Akt/p300 and PLC/Ca<sup>2+</sup>/calmodulin/CaMKII/HDAC4 signaling pathways in HTSMCs. Our findings, together with previous observations, suggest that elevated VCAM-1 expression is involved in causing PMNs migration and inflammation responses.

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## References

- Backs J, Song K, Bezprozvannaya S, Chang S, and Olson EN (2006) CaM Kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. *J Clin Invest* **116**:1853-1864.
- Barnes PJ, Adcock IM, and Ito K (2005) Histone acetylation and deacetylation: Importance in inflammatory lung diseases. *Eur Respir J* **25**:552-563.
- Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, Bradding P, Brightling CE, Wardlaw AJ, and Pavord ID (2006) Evidence of a role of tumor necrosis factor- $\alpha$  in refractory asthma. *N Engl J Med* **354**:697-708.
- Chan HM and La Thangue NB (2001) p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci* **114**:2363-2373.
- de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, and van Kuilenburg AB (2003) Histone deacetylases (HDACs): Characterization of the classical HDAC family. *Biochem J* **370**:737-749.
- Deng WG, Zhu Y, Wu KK (2003). Up-regulation of p300 binding and p50 acetylation in tumor necrosis factor- $\alpha$ -induced cyclooxygenase-2 promoter activation. *J Biol Chem* **278**:4770-4777.
- Dynan WS and Tjian R (1983) The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* **35**:79-87.
- Guha M and Mackman N (2002) The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* **277**:32124-32132.
- Han S, Lu J, Zhang Y, Cheng C, Han L, Wang X, Li L, Liu C, and Huang B (2006) Recruitment of histone deacetylase 4 by transcription factors represses interleukin-5 transcription. *Biochem J* **400**:439-448.
- Hsieh HL, Wang HH, Wu CY, Jou MJ, Yen MH, Parker P, and Yang CM (2007) BK-induced COX-2 expression via PKC- $\delta$ -dependent activation of p42/p44 MAPK and NF- $\kappa$ B in astrocytes. *Cell Signal* **19**:330-340.
- Hsieh HL, Wu CY, Hwang TL, Yen MH, Parker P, and Yang CM (2005) BK-induced cytosolic phospholipase A<sub>2</sub> expression via sequential PKC- $\delta$ , p42/P44 MAPK, and NF- $\kappa$ B activation in rat brain astrocytes. *J Cell Physiol* **206**:246-254.
- Huang L (2006) Targeting histone deacetylases for the treatment of cancer and inflammatory diseases. *J Cell Physiol* **209**:611-616.
- Huang WC and Chen CC (2005) Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. *Mol Cell Biol* **25**:6592-6602.
- Huang WC, Chen JJ, and Chen CC (2003) c-Src-dependent tyrosine phosphorylation of IKK $\beta$  is involved in tumor necrosis factor- $\alpha$ -induced intercellular adhesion molecule-1

MOL 38091

- expression. *J Biol Chem* **278**:9944-9952.
- Huang WC, Chen JJ, Inoue H, and Chen CC (2003) Tyrosine phosphorylation of I- $\kappa$ B Kinase  $\alpha/\beta$  by protein kinase C-dependent c-Src activation is involved in TNF- $\alpha$ -induced cyclooxygenase-2 expression. *J Immunol* **170**:4767-4775.
- Hung JJ, Wang YT and Chang WC (2006) Sp1 deacetylation induced by phorbol ester recruits p300 to activate 12(S)-lipoxygenase gene transcription. *Mol Cell Biol* **26**:1770-1785.
- Impey S, Fong AL, Wang Y, Cardinaux JR, Fass DM, Obrietan K, Wayman GA, Storm DR, Soderling TR, and Goodman RH (2002) Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM Kinase IV. *Neuron* **34**:235-244.
- Inoue K, Kobayashi M, Yano K, Miura M, Izumi A, Mataka C, Doi T, Hamakubo T, Reid PC, Hume DA, Yoshida M, Aird WC, Kodama T, and Minami T (2006) Histone deacetylase inhibitor reduces monocyte adhesion to endothelium through the suppression of vascular cell adhesion molecule-1 expression. *Arterioscler Thromb Vasc Biol* **26**:2652-2659.
- Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, and Barnes PJ (2005) Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N Engl J Med* **352**:1967-1976.
- Kadonaga JT, Carner KR, Masiarz FR, and Tjian R (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**:1079-1090.
- Koyasu S (2003) The role of PI3K in immune cells. *Nat Immunol* **4**:313-319.
- Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, and Lee YC (2003) Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* **111**:1083-1092.
- Lee CW, Chien CS, and Yang CM (2004) Lipoteichoic acid-stimulated p42/p44 MAPK activation via Toll-Like Receptor 2 in tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **286**:L921-L930.
- Lee CW, Lin WN, Lin CC, Luo SF, Wang JS, Pouyssegur J, and Yang CM (2006) Transcriptional regulation of VCAM-1 expression by tumor necrosis factor- $\alpha$  in human tracheal smooth muscle cells: Involvement of MAPKs, NF- $\kappa$ B, p300, and histone acetylation. *J Cell Physiol* **207**:174-186.
- Lee KS, Jin SM, Kim HJ, and Lee YC (2003) Matrix metalloproteinase inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* **111**:1278-1284.
- Lee KS, Lee HK, Hayflick JS, Lee YC, and Puri KD (2006) Inhibition of phosphoinositide 3-Kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J* **20**:455-465.

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- Lin HY, Chen CS, Lin SP, Weng JR, and Chen CS (2006) Targeting histone deacetylase in cancer therapy. *Med Res Rev* **26**:397-413.
- Little GH, Bai Y, Williams T, and Poizat C (2007) Nuclear calcium/calmodulin-dependent protein kinase II $\delta$  preferentially transmits signals to histone deacetylase 4 in cardiac cells. *J Biol Chem* **282**:7219-7231.
- Liu Y, Randall WR, and Schneider MF (2005) Activity-dependent and -independent nuclear fluxes of HDAC4 mediated by different kinases in adult skeletal muscle. *J Cell Biol* **168**:887-897.
- Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I (1998). The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF- $\kappa$ B by directly targeting p65. *J Biol Chem* **273**:33508-33516.
- Ma Z, Chang MJ, Shah RC, and Benveniste EN (2005) Interferon- $\gamma$ -activated STAT-1 $\alpha$  suppresses MMP-9 gene transcription by sequestration of the coactivators CBP/p300. *J Leukoc Biol* **78**(2), 515-523.
- Marganski WA, Gangopadhyay SS, Je HD, Gallant C, and Morgan KG (2005) Targeting of a novel Ca<sup>+2</sup>/Calmodulin-dependent protein kinase II is essential for extracellular signal-regulated kinase-mediated signaling in differentiated smooth muscle cells. *Circ Res* **97**:541-549.
- Min JK, Kim YM, Kim SW, Kwon MC, Kong YY, Hwang IK, Won MH, Rho J, and Kwon YG (2005) TNF-related activation-induced cytokine enhances leukocyte adhesiveness: Induction of ICAM-1 and VCAM-1 via TNF receptor-associated Factor and protein kinase C-dependent NF- $\kappa$ B activation in endothelial cells. *J Immunol* **175**:531-540.
- Mukhopadhyay S, Hoidal JR, and Mukherjee TK (2006) Role of TNF $\alpha$  in pulmonary pathophysiology. *Respir Res* **7**:125-134.
- Neish AS, Khachigian LM, Park A, Baichwal VR, and Collins T (1995) Sp1 is a component of the cytokine-inducible enhancer in the promoter of vascular cell adhesion molecule-1. *J Biol Chem* **270**:28903-28909.
- Nie M, Pang L, Inoue H, and Knox AJ (2003) Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1 $\beta$  in human airway smooth muscle cells: Involvement of different promoter elements, transcription factors, and histone H4 acetylation. *Mol Cell Biol* **23**:9233-9244.
- Poizat C, Puri PL, Bai Y, and Kedes L (2005) Phosphorylation-dependent degradation of p300 by doxorubicin-activated p38 mitogen-activated protein kinase in cardiac cells. *Mol Cell Biol* **25**:2673-2687.
- Rose JL, Huang H, Wray SF, and Hoyt DG (2005) Integrin engagement increases histone H3 acetylation and reduces histone H1 association with DNA in murine lung endothelial cells.

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*Mol Pharmacol* **68**:439-446.

Roth SY, Denu JM, and Allis CD (2001) Histone acetyltransferases. *Annu Rev Biochem* **70**:81-120.

Sato K, Suematsu A, Nakashima T, Takemoto-Kimura S, Aoki K, Morishita Y, Asahara H, Ohya K, Yamaguchi A, Takai T, Kodama T, Chatila TA, Bito H, and Takayanagi H (2007) Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. *Nat Med* **12**:1410-1416.

Soderling TR, Chang B, and Brickey D (2001) Cellular signaling through multifunctional Ca<sup>2+</sup>/Calmodulin-dependent protein kinase II. *J Biol Chem* **276**:3719-3722.

Tong Q, Zheng L, Lin L, Li B, Wang D, and Li D (2006) Hypoxia-induced mitogenic factor promotes vascular adhesion molecule-1 expression via the PI-3K/Akt-NF-κB signaling pathway. *Am J Respir Cell Mol Biol* **35**:444-456.

Wang CC, Lin WN, Lee CW, Lin CC, Luo SF, Wang JS, and Yang C M (2005) Involvement of p42/p44 MAPK, p38 MAPK, JNK, and NF-κB in IL-1β-induced VCAM-1 expression in human tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **288**:L227-L237.

Yang CM, Hsieh HL, and Lee CW (2005) Intracellular signaling mechanisms underlying the expression of pro-inflammatory mediators in airway diseases. *Chang Gung Med J* **28**:813-823.

Yuan LW and Gambée JE (2000) Phosphorylation of p300 at serine 89 by protein kinase C. *J Biol Chem* **275**:40946-40951.

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## Figure legends

**Fig. 1.** Inhibition of Src, EGFR, and PI3K/Akt prevents TNF- $\alpha$ -induced neutrophil adhesion to HTSMCs and VCAM-1 expression. HTSMCs (~80% confluence in 12-well plates) were transfected with control siRNA or Src and Akt siRNA (100 nM) for 72 h as described under "Experimental Procedures" or pretreated with inhibitors of Src (PP1), EGFR (AG1478), and PI3K (LY294002 and wortmannin) at 10  $\mu$ M for 1 h and then challenged with TNF- $\alpha$  for (A) 24 h and (B) 4 h. (A) The cell lysates were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane and then blotted using an antiserum reactive with VCAM-1 or GAPDH (as an internal control) antibody as described in Materials and Methods. (B) The isolated RNA samples were analyzed by RT-PCR, using the primers specific for VCAM-1 and  $\beta$ -actin, respectively. (C) TNF- $\alpha$ -stimulated Akt phosphorylation, cells were pretreated with PP1, AG1478, and LY294002 for 1 h or transfected with siRNAs (Src and Akt) and then stimulated with 30 ng/ml TNF- $\alpha$  for 30 min. Whole cell lysates were subjected to 12% SDS-PAGE, transferred to nitrocellulose membrane and then blotted using an antiserum reactive with anti-phospho-Akt or GAPDH (as an internal control) antibody. Results are one representative of three independent experiments. (D) Neutrophils, labeled with BCECF, were added to HTSMCs incubated with TNF- $\alpha$  for 24 h in the presence of PP1, AG1478 and LY294002, and then the adhesion was measured in the absence or presence of anti-VCAM-1 antibody (1:100 dilution) as described in Fig. 1A. Data are expressed as mean  $\pm$  SEM of three independent experiments. <sup>#</sup>p<0.01 as compared with the cells exposed to TNF- $\alpha$  alone.

**Fig. 2.** Recruitment of NF- $\kappa$ B, p300 and Akt to VCAM-1 promoter in response to TNF- $\alpha$ . (A) Neutrophils, labeled with BCECF, were added to HTSMCs pretreated with 10  $\mu$ M curcumin for 1 h before incubation with TNF- $\alpha$  for 24 h, and then the incubation was continued at 37°C for 1 h. The adhesion was measured as described in Fig. 1A. Data are expressed as mean  $\pm$  SEM of three independent experiments. \*p<0.05 as compared with the cells exposed to TNF- $\alpha$  alone. (B) Cells were pretreated with 10  $\mu$ M of curcumin or MG132 for 1 h and then incubated with TNF- $\alpha$  for 4 h. The isolated RNA samples were analyzed by RT-PCR as described in Fig. 1. (C) HTSMCs (~80% confluence in 12-well plates) were transfected with p300 siRNA (100 nM) for 72 h as described under "Experimental Procedures" and then challenged with TNF- $\alpha$  for 24 h. (D) Nuclear Akt and p65 co-immunoprecipitated with anti-p300 antibody, cells were pretreated with LY294002, curcumin and helenalin at 10  $\mu$ M for 1 h before incubation with TNF- $\alpha$  for 2 h. Equal amounts (1 mg) of nuclear extracts were immunoprecipitated (IP) with an anti-p300 Ab, separated by 10% SDS-PAGE, and immunoblotted with anti-phospho-Akt, anti-p50 or anti-p65 Ab as indicated. (E, F) In ChIP

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assay, the sequence of the human VCAM-1 promoter region (-403/-30) was amplified by PCR primer pairs. An enrichment of the VCAM-1 promoter DNA was shown after PCR amplification of immunoprecipitates of p300- and histone H4-associated DNA from cells treated with TNF- $\alpha$  for various times. Confluent and serum-starved HTSMCs in 10-cm dishes were pretreated with LY294002 or helenalin (HLN) for 1 h before incubation with 30 ng/ml TNF- $\alpha$  for 2 h. The in vivo protein-DNA complexes were cross-linked by formaldehyde treatment and chromatin pellets were extracted and sonicated. The associated VCAM-1 promoter DNA was amplified by PCR as described in Materials and Methods. The input represents PCR products from chromatin pellets prior to immunoprecipitation.

**Fig. 3.** Inhibition of PLC, Ca<sup>2+</sup>/calmodulin and CaMKII prevents TNF- $\alpha$ -induced neutrophil adhesion to HTSMCs and VCAM-1 expression. Cells were pre-incubated with inhibitors of PI-PLC (U73122, 1  $\mu$ M), Ca<sup>2+</sup> (BAPTA/AM, 0.3  $\mu$ M), CaMKII (KT5926, 1  $\mu$ M), and CaM (calmidazolium chloride, 1  $\mu$ M) for 1 h and then incubated with TNF- $\alpha$  for (A) 24 h and (B) 4 h. (A) The cell lysates were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane to determine the level VCAM-1 protein expression as described in Fig. 1. Data are expressed as mean  $\pm$  SEM of three independent experiments. #p<0.01 as compared with the cells exposed to TNF- $\alpha$  alone. (B) The isolated RNA samples were analyzed by RT-PCR, using the primers specific for VCAM-1 and  $\beta$ -actin, respectively. (C) Cells were pretreated with various concentrations of KN62 (a CaMKII inhibitor) for 1 h and then incubated with TNF- $\alpha$  for 24 h. (D) TNF- $\alpha$ -stimulated CaMKII phosphorylation, cells were pretreated with U73122, BAPTA/AM, calmidazolium chloride, and KN62 for 1 h and then stimulated with 30 ng/ml TNF- $\alpha$  for the indicated time. Whole cell lysates were subjected to 12% SDS-PAGE, transferred to nitrocellulose membrane, and then blotted using an antiserum reactive with anti-phospho-CaMKII Ab. Membranes were stripped and re-probed with anti-GAPDH Ab as an internal control. Results are one representative of three independent experiments. (E) HTSMCs were treated with 30 ng/ml TNF- $\alpha$  for the indicated times or pretreated with KN62 (10  $\mu$ M) for 1 h and then stimulated with 30 ng/ml TNF- $\alpha$  for 10 min. Whole cell lysates were immunoprecipitated with an anti-CaMKII Ab for overnight at 4°C. The immunoprecipitated proteins were diluted with kinase buffer and analyzed by a CaMKII assay kit (Upstate). Data are expressed as mean  $\pm$  SEM of three independent experiments. #p<0.05, as compared with untreated basal. \*p<0.05, as compared with the cells exposed to TNF- $\alpha$  alone for 10 min. The basal level activity of CaMKII was ranged from 1.58 to 3.08X10<sup>2</sup> pmol/min/mg protein. (F) Neutrophils, labeled with BCECF, were added to HTSMCs pretreated with KT5926 and calmidazolium chloride for 1 h before incubation with TNF- $\alpha$  for 24 h, and continued incubation at 37°C for 1 h. The adhesion was measured as

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described in Materials and Methods. Data are expressed as mean  $\pm$  SEM of three independent experiments. <sup>#</sup>p<0.01 as compared with the cells exposed to TNF- $\alpha$  alone.

**Fig. 4.** HDAC4 is recruited to the VCAM-1 promoter regions and its nucleo-cytoplasm shuttling is regulated by CaMKII. Time dependence of TNF- $\alpha$ -induced HDAC4 exportation from nucleus to cytosol, cells were treated with 30 ng/ml TNF- $\alpha$  for various times (A) or in the presence of calmidazolium chloride and KT5926 at 10  $\mu$ M for 1 h and then treated with TNF- $\alpha$  for 60 min (B). Cells were harvested and centrifuged to prepare cytosolic and nuclear fractions. The resultant fractions were subjected to 10% SDS-PAGE and analyzed using an anti-HDAC4 antibody as described in Fig. 1. (C) Cells were stimulated with 30 ng/ml TNF- $\alpha$  for various times or pretreated with KN62 for 1 h before incubation with TNF- $\alpha$  for 1 h. Cells were then fixed and labeled with anti-HDAC4 Ab and a fluorescein isothiocyanate (FITC)-conjugated secondary Ab. Individual cells were imaged as described in Methods. Image represents one of three individual experiments. (D) An enrichment of the VCAM-1 promoter DNA was shown after PCR amplification of immunoprecipitates of HDAC4-associated DNA from cells treated with TNF- $\alpha$  for various times. Confluent and serum-starved HTSMCs in 10-cm dishes were incubated with TNF- $\alpha$  (30 ng/ml) for the indicated times or pretreated with KT5926 for 1 h before incubation with TNF- $\alpha$  for 1 h. The ChIP assay was performed as described in Fig. 2. (E) Cells (~80% confluence in 12-well plates) were transfected with control siRNA and HDAC4 siRNA (100 nM) for 72 h as described under "Experimental Procedures" or pretreated with TSA for 1 h and then incubated with TNF- $\alpha$  for 24 h. The cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane to determine the level VCAM-1 protein expression as described in Fig. 1. Results represent one of three individual experiments. (F) Neutrophils, labeled with BCECF, were added to HTSMCs pretreated with KT5926 and TSA for 1 h before incubation with TNF- $\alpha$  for 24 h, and continued incubation at 37°C for 1 h. The adhesion was measured as described in Materials and Methods. Data are expressed as mean  $\pm$  SEM of three independent experiments. <sup>#</sup>p<0.01 as compared with the cells exposed to TNF- $\alpha$  alone.

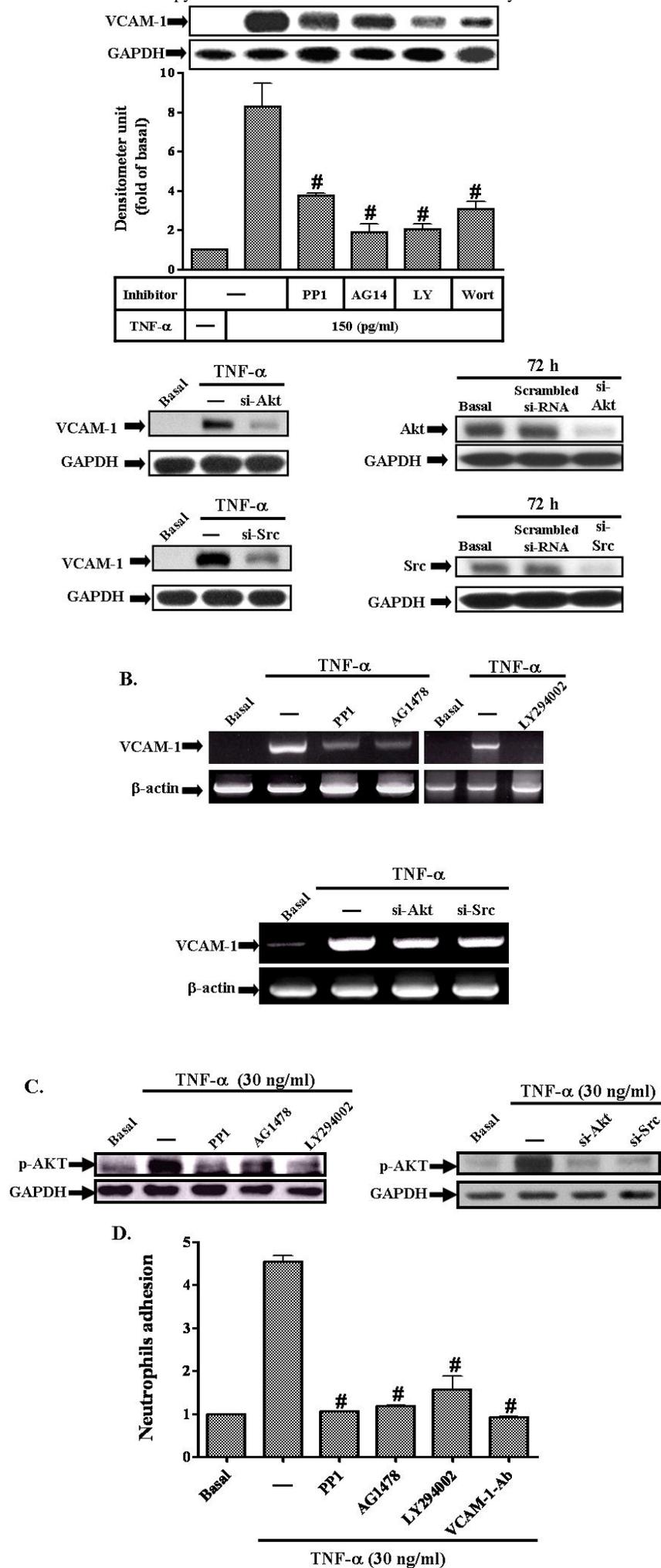
**Fig. 5.** Involvement of PKC isoforms in TNF- $\alpha$ -induced VCAM-1 expression. (A) HTSMCs were pretreated with GÖ6976, rottlerin and RO318220 at 10  $\mu$ M for 1 h and then incubated with 150 pg/ml TNF- $\alpha$  for 24 h. The cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane to determine the level of VCAM-1 expression as described in Fig. 1. Data are expressed as mean  $\pm$  SEM of three independent experiments. <sup>#</sup>p<0.01 as compared with the cells exposed to TNF- $\alpha$  alone. (B) Time dependence of TNF- $\alpha$ -stimulated translocation of PKC isoforms from cytosol to cell membrane, cells were

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treated with 30 ng/ml TNF- $\alpha$  for various times or with PMA for 24 h. Cells were harvested and centrifuged to prepare cytosolic and membrane fractions. The resultant fractions were subjected to 10% SDS-PAGE and analyzed with anti-PKC isoforms antibodies as described in Fig. 1. To confirm the involvement of PKC isoforms in VCAM-1 expression, cells were transfected with dominant negative mutants of PKC $\alpha$  and PKC $\iota$  and then incubated with TNF- $\alpha$  for (C) 24 h and (D) 4 h. (C) The cell lysates were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane to determine the level of VCAM-1 expression as described in Fig. 1. Results represent one of three individual experiments. (D) The isolated RNA samples were analyzed by RT-PCR, using the primers specific for VCAM-1 and  $\beta$ -actin, respectively. (E) HTSMCs (~80% confluence in 12-well plates) were transfected with siRNA for PKC $\alpha$  and PKC $\mu$  (100 nM) for 72 h as described under "Experimental Procedures" and then challenged with TNF- $\alpha$  for 24 h. The cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane to determine the level of VCAM-1, PKC $\alpha$  and PKC $\mu$  expression as described in Fig. 1. Results represent one of three individual experiments.

**Fig. 6.** Inhibition of Src/EDGFR/PI3K/Akt and PLC/Ca<sup>2+</sup>/camodulin/CaMKII pathways attenuates TNF- $\alpha$ -induced VCAM-1 promoter activity. Cells transiently transfected with VCAM-1 reporter gene were (A) treated with TNF- $\alpha$  (30 ng/ml) for various times and (B) pretreated with PP1, AG1478, LY294002, U73122, BAPTA/AM, calmidazolium chloride, KT5926 and GM6001 for 1 h before incubation with TNF- $\alpha$  for 4 h. Luciferase activity was assayed as described in Materials and Methods. Data are expressed as mean  $\pm$  SEM of three independent experiments. \*p<0.05, #p<0.01 as compared with the cells exposed to vesicle (A) or TNF- $\alpha$  alone (B).

**Fig. 7.** Schematic pathways illustrated that TNF- $\alpha$ -mediated VCAM-1 induction was indeed governed by transcript-specific signaling pathway, involving the Src/EGFR/PI3K and PLC/Ca<sup>2+</sup>/CaM. Phosphorylation of Akt and CaM kinase II by these two pathways may eventually lead to enhance the activity of HAT and HDAC. Finally, the elevated VCAM-1 expression on HTSMCs is involved in causing PMNs migration and airway inflammatory responses.



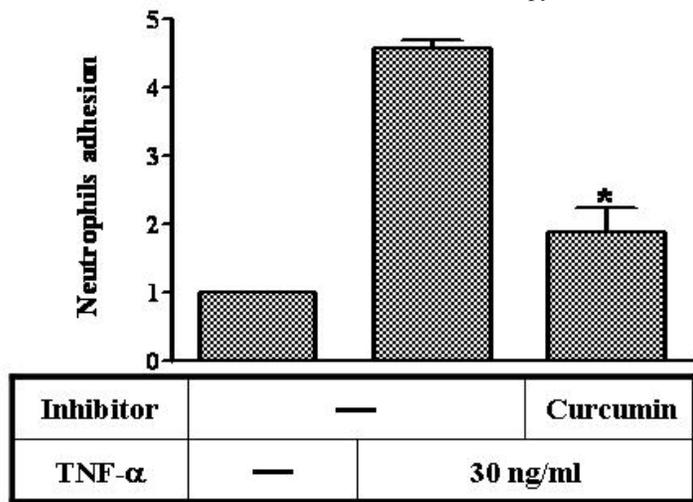
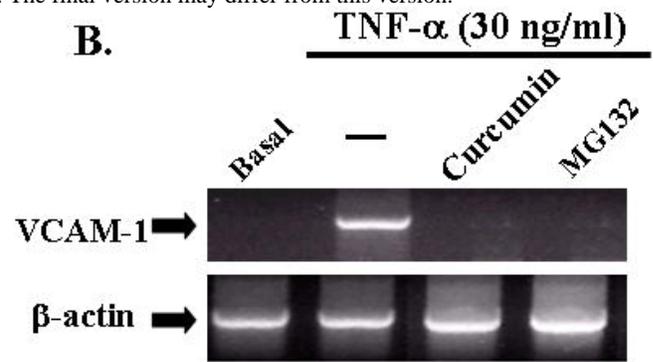
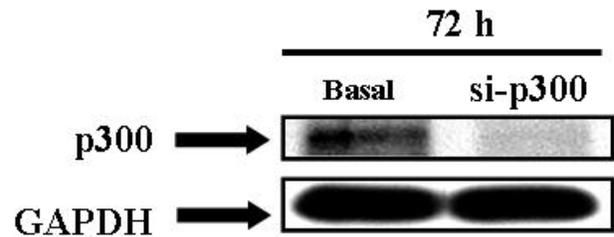
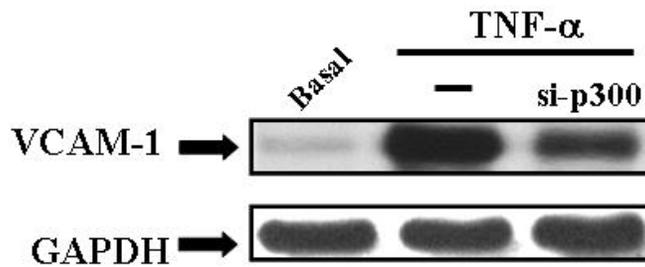
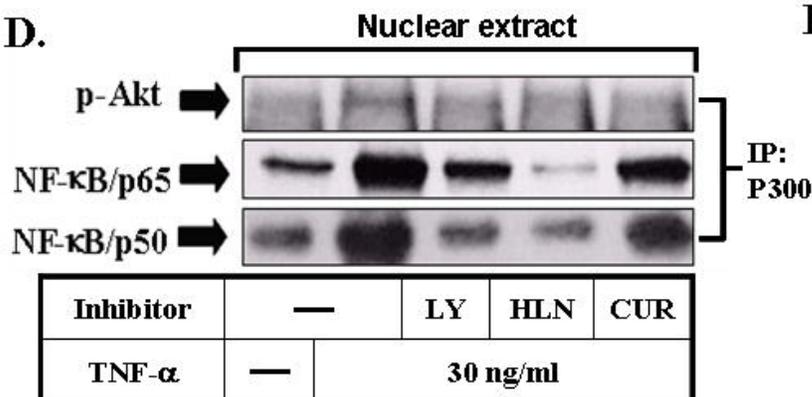
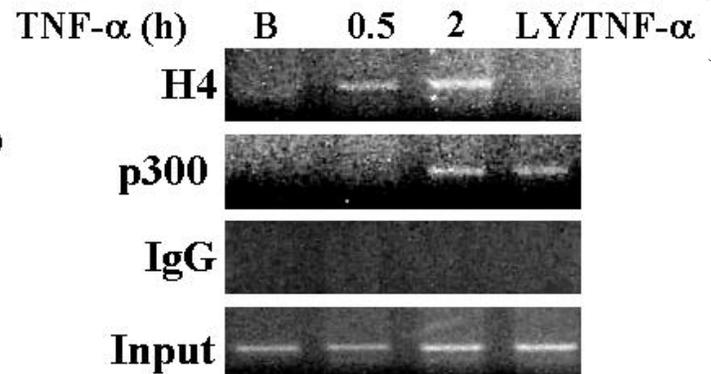
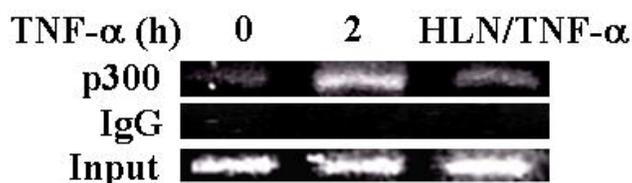
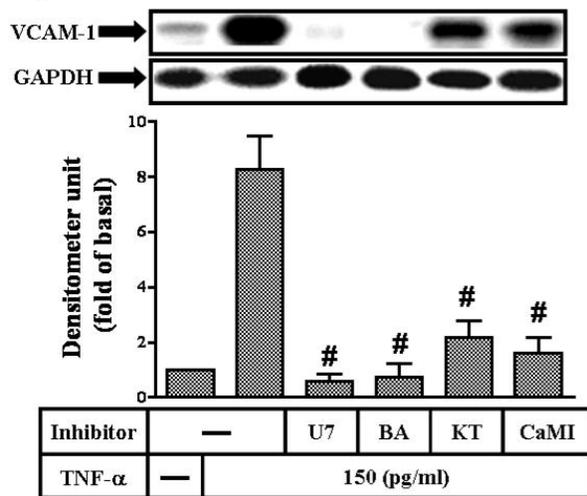
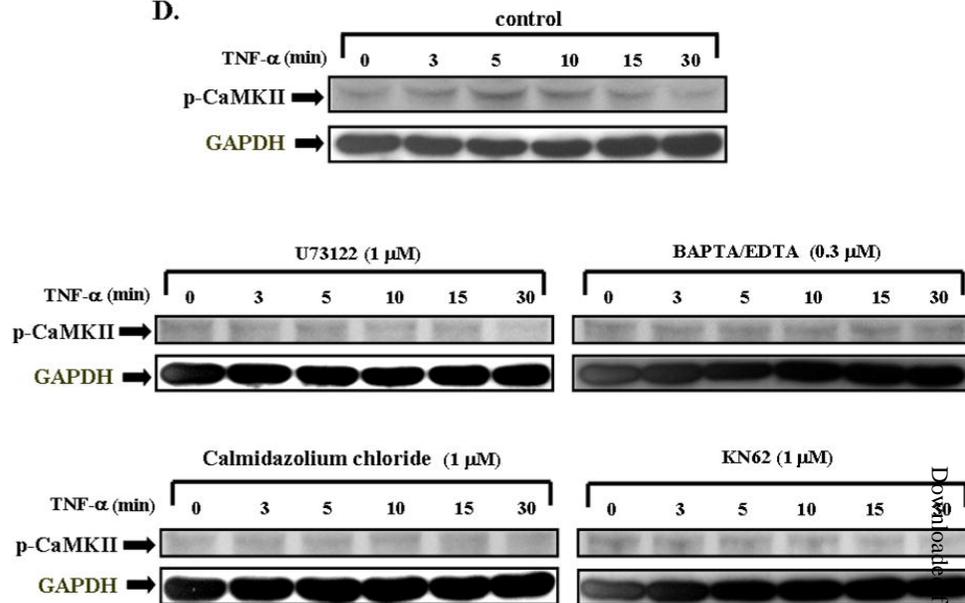
**Fig 2A.****B.****C.****D.****E.****F.**

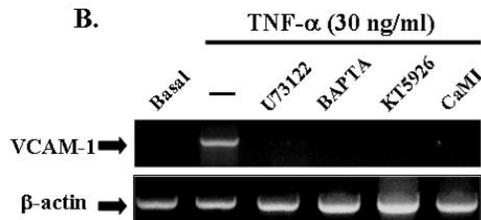
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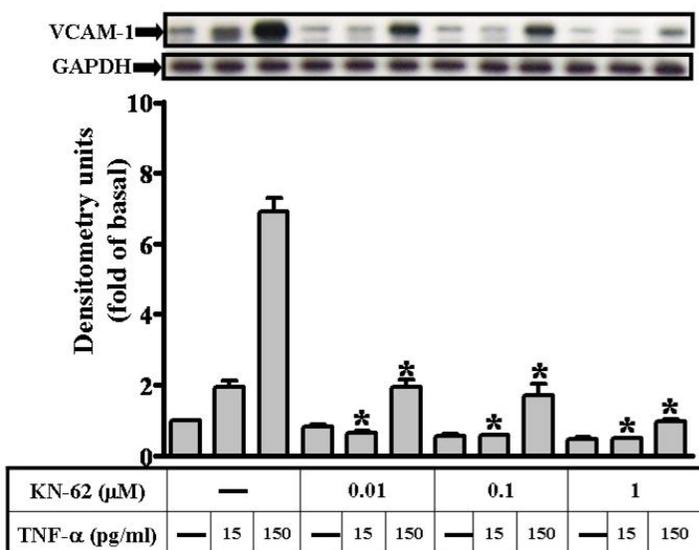
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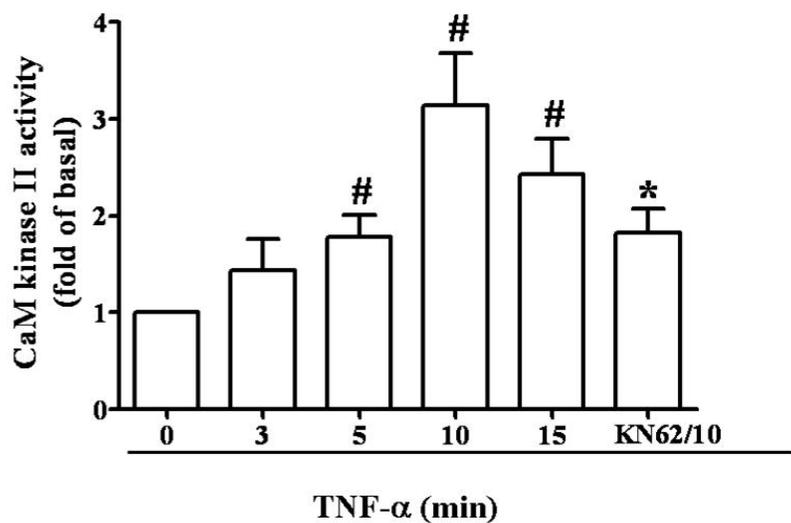
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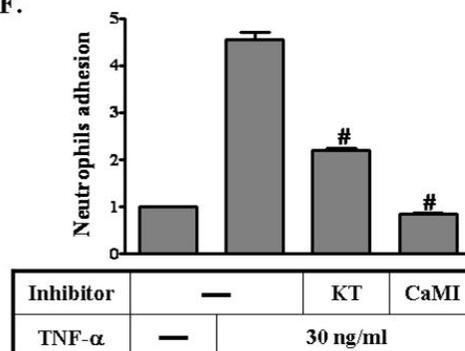
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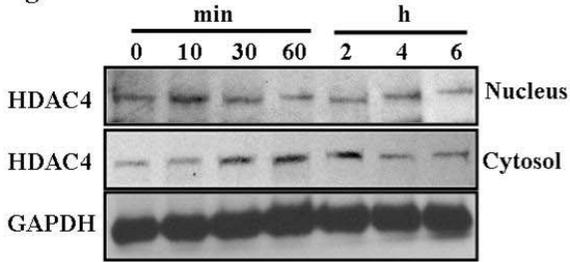
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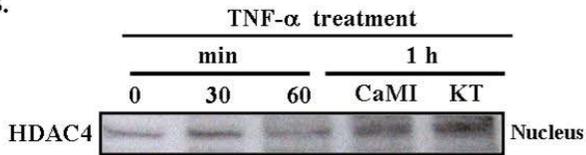
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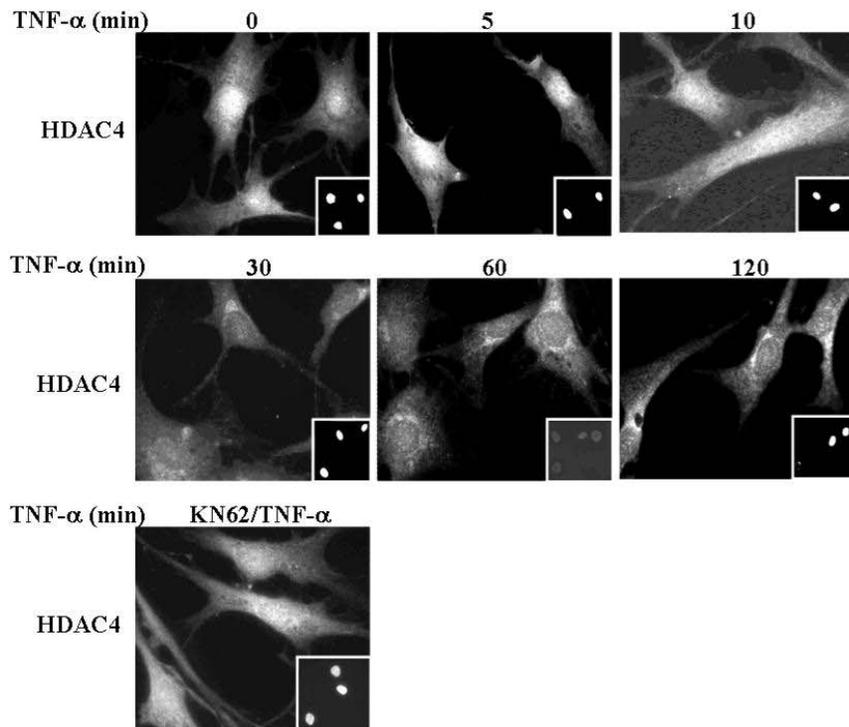
**Fig 4A.**



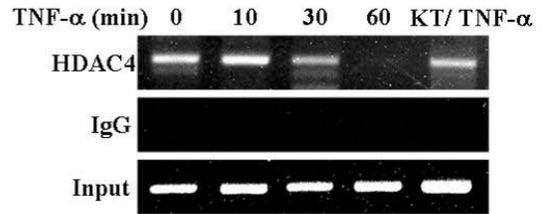
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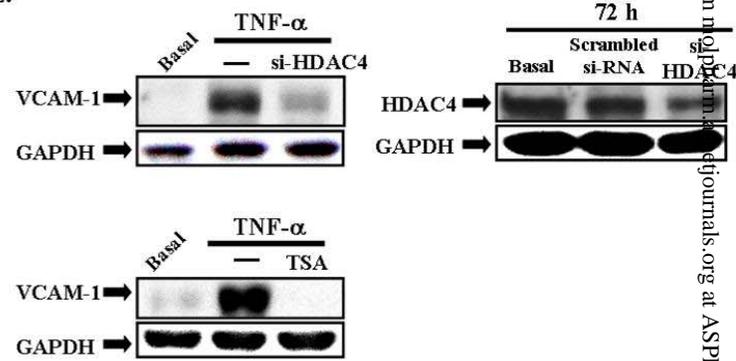
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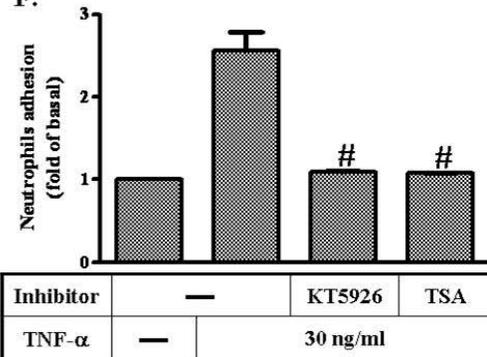
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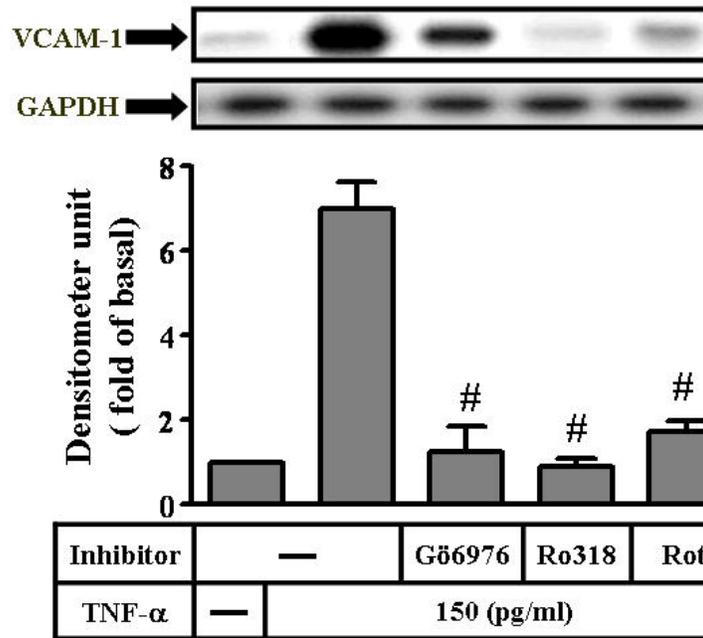


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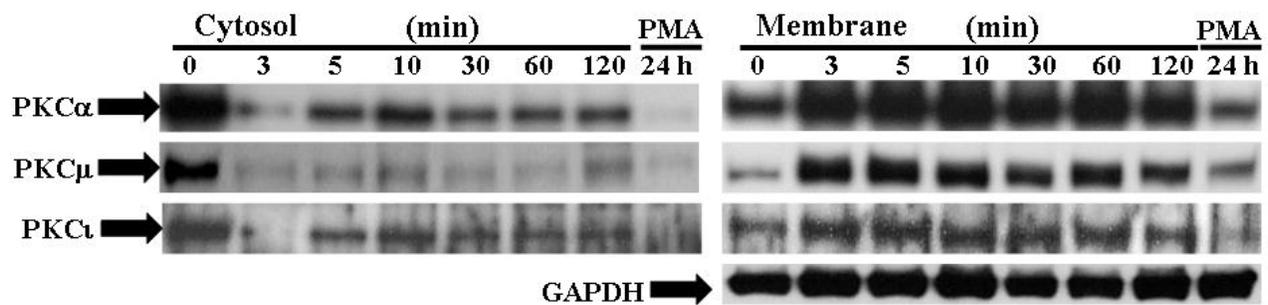


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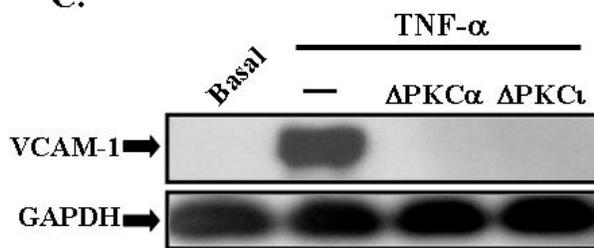




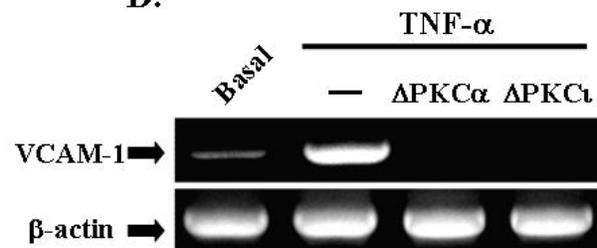
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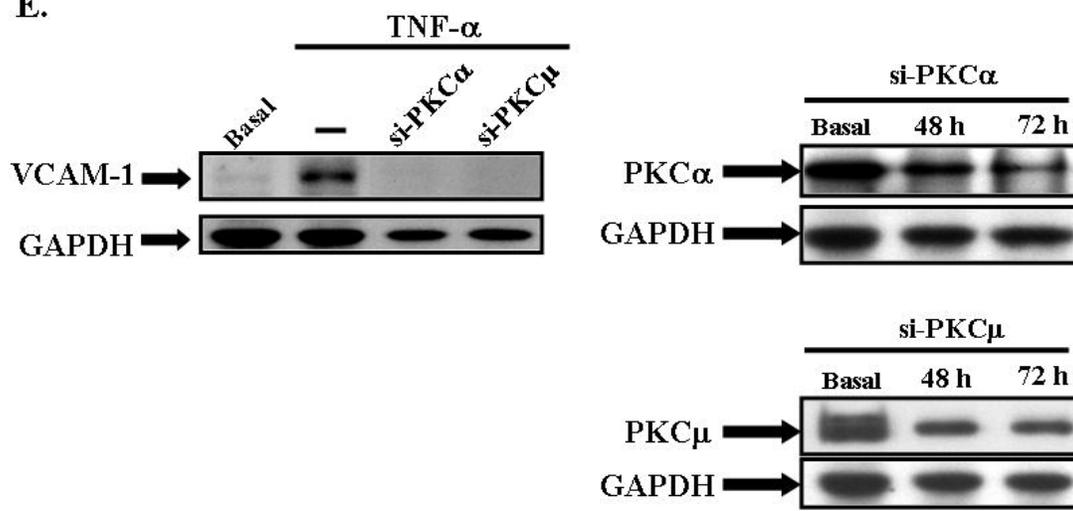
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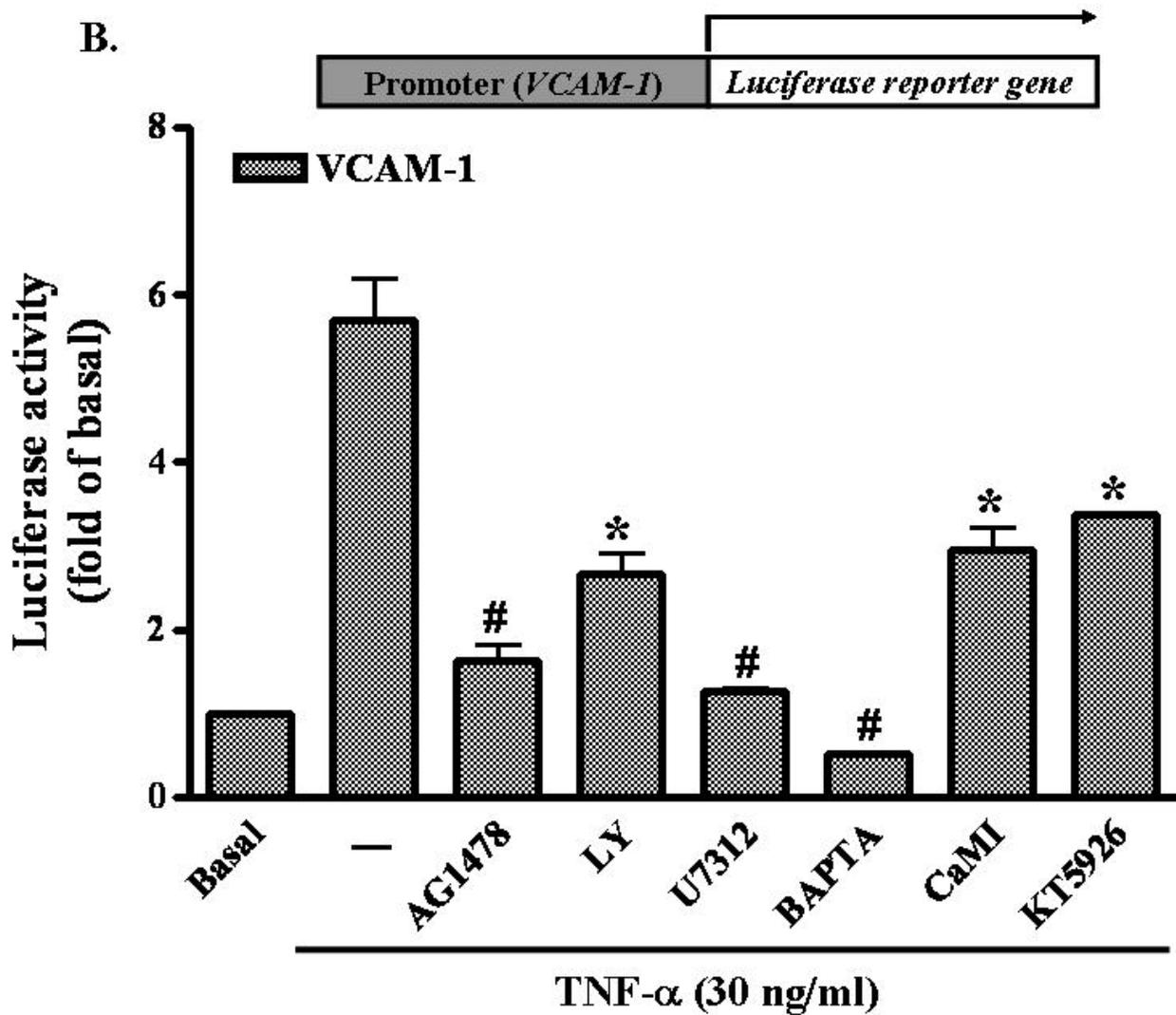
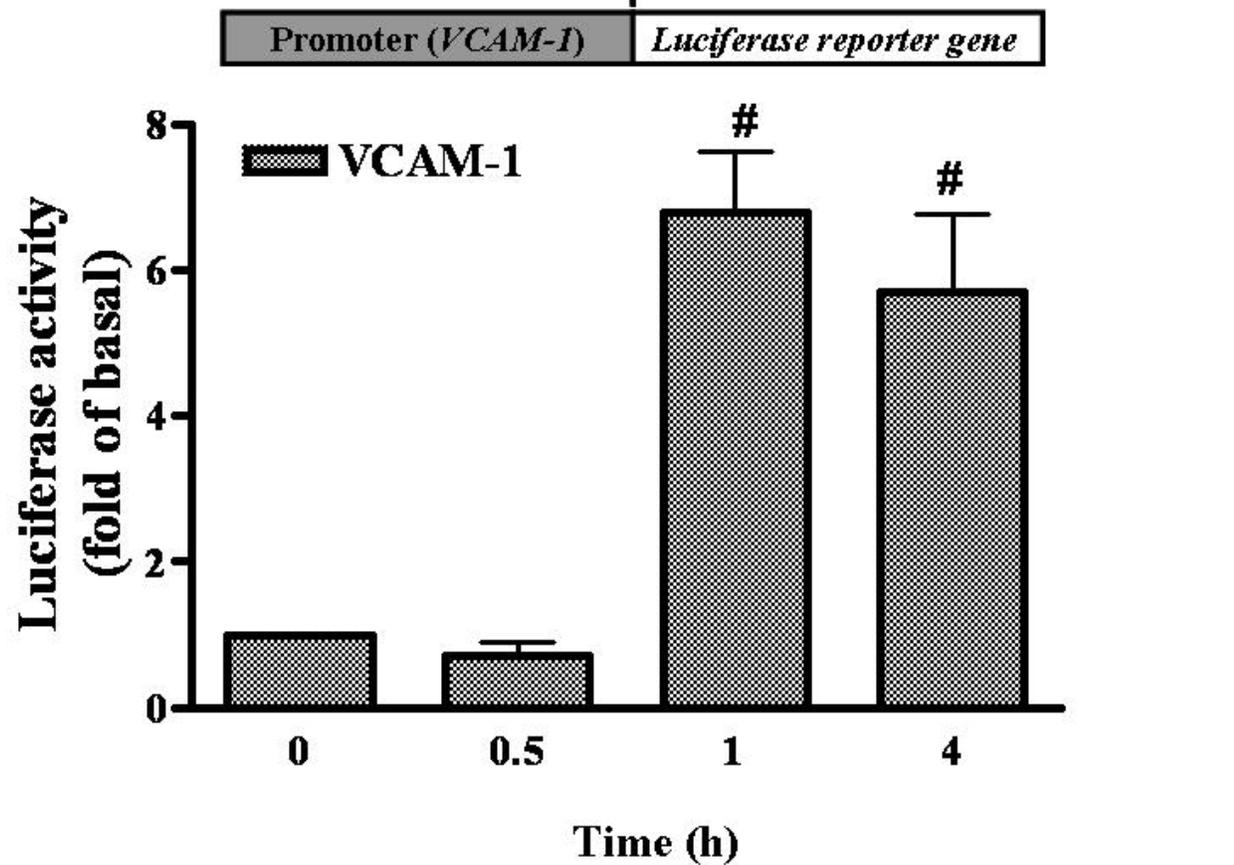


Fig.7.

