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Rac1 and Superoxide Are Required for the Expression of Cell Adhesion Molecules Induced by TNF-α in Endothelial Cells

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Running head: Rac1 and expression of endothelial cell adhesion molecules

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Selected abbreviations and acronyms

CAT = chloramphenicol acetyltransferase.

HAEC = human aortic endothelial cells

HMEC = human microvascular endothelial cells

ICAM-1 = intercellular cell adhesion molecule-1

ROS = reactive oxygen species

SOD = superoxide dismutase

TNF-α = tumor necrosis factor-α

VCAM-1 = vascular cell adhesion molecule-1

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Abstract

Oxidative signals play an important role in the regulation of endothelial cell adhesion molecule expression. Small GTP-binding protein Rac1 is activated by various proinflammatory substances and regulates superoxide generation in endothelial cells (EC). In the present study, we demonstrate that adenoviral-mediated expression of dominant negative N17Rac1 (Ad.N17Rac1) suppresses TNF-α induced vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin gene expression in a dose dependent manner. Ad.N17Rac1 did not inhibit TNF-α induced activation of nuclear NF-κB binding activity or IκB-α degradation. In contrast, Ad.N17Rac1 inhibited TNF-α induced NF-κB-driven HIV$(κB)_4$CAT and p288VCAM-Luc promoter activity, suggesting that N17Rac1 inhibits TNF-α induced VCAM-1, E-selectin and ICAM-1 through suppressing NF-κB mediated transactivation. In addition, expression of superoxide dismutase by adenovirus suppressed TNF-α induced VCAM-1, E-selectin and ICAM-1 mRNA accumulation. However, adenoviral mediated expression of catalase only partially inhibited TNF-α induced E-selectin gene expression, and had no effect on VCAM-1 and ICAM-1 gene expression. These data suggest that Rac1 and superoxide play crucial roles in the regulation of expression of cell adhesion molecules in endothelial cells.

Key words: Rac1, superoxide, TNF-α, vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, NF-κB, endothelial cells.

Expression of cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin on endothelial cells represents one of the earliest pathological changes in immune and inflammatory diseases such as atherosclerosis (Springer, 1995). Induction of these molecules by tumor necrosis factor-α (TNF-α) and other inflammatory cytokines is regulated at the level of gene transcription and requires binding of the transcription factor NF-κB to the regulatory region within the promoters of each of these genes (Collins et al., 1995). NF-κB is an ubiquitous transcription factor that induces the expression of a variety of inflammatory and immune genes (Baeuerle and Henkel, 1994). In most cells, NF-κB is retained in the cytoplasm in an inactive complex with the inhibitor IκB. Inflammatory agents such as cytokines cause phosphorylation of IκB followed by its degradation through the ubiquitin-proteasome pathway. NF-κB is then dissociated from IκB and translocated to the nucleus, where it binds to promoter recognition sites (Baeuerle and Henkel, 1994).

Oxidative signals play an important role in the regulation of inflammatory gene expression in endothelial cells (Marui et al., 1993; Chen and Medford, 1999). VCAM-1 and monocyte chemoattractant protein-1 (MCP-1) gene expression by diverse inflammatory signals occurs through an oxidation reduction (redox)-sensitive mechanism via NF-κB (Marui et al., 1993; Chen and Medford, 1999). Inflammatory stimuli-activated endothelial expression of VCAM-1 and MCP-1 is inhibited by antioxidants such as pyrolidine dithiocarbamate (Marui et al., 1993; Chen and Medford,

1999). The NADPH oxidase inhibitor, diphenylene iodonium, suppresses TNF-α induced superoxide ($O_2^{-\cdot}$) generation and VCAM-1 gene expression in endothelial cells (Tummala et al., 2000). In contrast, reactive oxygen species (ROS) such as H_2O_2 stimulate MCP-1 gene expression in vascular smooth muscle cells (Chen et al., 1998). However, the role of specific ROS, such as $O_2^{-\cdot}$ and H_2O_2, in the regulation of the expression of inflammatory genes remains unclear.

Rac1 is a member of the Rho family of small GTPases involved in signal transduction pathways that control proliferation, adhesion, and migration of cells during embryonic development and invasiveness of tumor cells (Matos et al., 2000). In phagocytic cells, Rac proteins are involved in the assembly of the neutrophil NADPH oxidase system and responsible for transferring electrons from NADPH to molecular oxygen with the subsequent production of $O_2^{-\cdot}$ (Abo et al., 1991). Many inflammatory signals including cytokines and lipopolysaccharide activate Rac1 (Sulciner et al., 1996a; Sulciner et al., 1996b). Rac1 functions as a regulator of ROS generation in a variety of non-phagocytic cells including endothelial cells (Sulciner et al., 1996a; Sulciner et al., 1996b). Recently, it has beens reported that Rac1 is involved in the activation of NF-κB (Sulciner et al., 1996a). Expression of constitutively active Rac1 (V12Rac1) results in an increase in $O_2^{-\cdot}$ generation in fibroblasts and activation of NF-κB in HeLa cells. Conversely, expression of dominant negative Rac1 inhibits $O_2^{-\cdot}$ generation by a variety of hormone stimuli, such TNF-α, IL-1β, and PDGF in fibroblasts. Inhibition of Rac1 also suppresses cytokine-stimulated NF-κB activation (Sulciner et al., 1996a; Sulciner et al., 1996b).

Since Rac1 regulates intracellular ROS generation and NF-κB activation in response to cytokines – two important signaling events in the up-regulation of cell adhesion molecules, we tested the hypothesis that Rac1 may be involved in cytokine-induced expression of cell adhesion molecules in endothelial cells. In this study we found that Rac1 is necessary for cytokine-induced expression of cell adhesion molecules. We further demonstrate that $O_2^{-\cdot}$, but not H_2O_2, is involved in the upregulation of VCAM-1, ICAM-1, and E-Selectin gene expression stimulated by TNF-α.

Materials and Methods

Cell Culture and DNA plasmids: Human aortic endothelial cells (HAEC) were obtained from Clonetics, Inc. and cultured in EGM-2 growth medium. Cells were used between passages 5 and 9. Human microvascular endothelial cells (HMEC) were described previously (Chen et al., 1997) and were cultured in modified MCDB 131 (Gibco BRL) supplemented with 10% fetal bovine serum and EGM Singlequots (Clonetics, Inc.). All cells were maintained at 37°C in a 5% CO_2 incubator. p288VCAM-Luc is a chimeric reporter construct containing coordinates –288 to 12 of the human VCAM-1 promoter, linked to a luciferase reporter gene. p(HIVκB)$_4$-CAT contains four tandem copies of HIV long terminal repeat κB DNA sequences linked to a chloramphenicol acetyl transferase (CAT) reporter gene (Kunsch et al., 1992). The empty expression vector pEXV and the myc epitope-tagged dominant negative (N17Rac1) expression vector were gifts from Dr. A. Hall (University College, London, United Kingdom) and have been previously described (Ridley et al., 1992).

Preparation of RNA and Northern blot analysis: Total cellular RNA was isolated by a single extraction with Tripure reagent (Boehringer Mannheim, Inc.) and size-fractionated using 1% agarose formaldehyde gels. RNA was transfered to nitrocellulose and hybridizations were performed as described previously (Chen et al., 1997). The cDNAs used were human VCAM-1, ICAM-1, E-selectin and GAPDH cDNA as described previously (Marui et al., 1993). Autoradiography was performed with a PhosphorImager 445sI (Molecular Dynamics, Sunnyvale, CA).

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Determination of cell surface expression of adhesion molecules by ELISA: HAEC were plated in 96-well plates and infected with recombinant adenovirus at indicated multiplicity of infection (MOI). Then cells were incubated with TNF-α (100 U/ml) for 16 hours. Primary mouse antibodies for VCAM-1, E-Selectin and ICAM-1 were obtained from Southern Biotechnology Associates. Cell surface expression of adhesion molecules was determined by primary binding with specific mouse antibodies, followed by secondary binding with a horseradish peroxidase-conjugated goat antimouse IgG antibody. Quantification was performed by determination of colorimetric conversion at OD450 nm of 3,3',5,5'-tetramethylbenzidine.

Transfection and assay of reporter gene activity: Since HAEC are relatively resistant to efficient transient transfection, we used HMEC for these experiments. HMEC were grown to 60 to 70 % confluence in 6 well plates and transfected with various plasmids as indicated in figure legends using SuperFect transfection reagent according to manufacturer's instructions (Qiagen, Inc). After a 24 hour recovery, HMEC were exposed to TNF-α (100 U/ml) for 16 hour. Then, protein extracts were prepared by rapid freeze-thaw in 0.25 M Tris, pH 8.0. For CAT activity assay, 5 μg of protein per sample was incubated with 5 μCi of ^{14}C labeled chloramphenicol (Amersham, Corp) and 5 μg of n-butyryl coenzyme A (Pharmacia Corp) for various times. The acetylated chloramphenicol forms were extracted using a 2:1 mixture of 2,6,10,14-tetra-methyl-pentadecane:xylenes and subjected to centrifuge (Kinston and Sheen, 1995). The organic phase was removed and counted to determine CAT activity. The pRL-TK

(renilla luciferase constitutively expressed under the control of thymidine kinase) was used to normalize transfection efficiency. All CAT activities were normalized against the renilla luciferase activity. Firefly and renilla luciferase activities were measured by using a luciferase reporter assay system according to the manufacturer's instructions (Promega, Inc.).

Nuclear extract preparation, ELISA or gel shift analysis of nuclear NF-κB binding activity: HAEC nuclear extracts were prepared as previously described (Tummala et al., 2000). Nuclear NF-κB binding activity was determined by using TransAM NF-κB p65 Transcriptional Factor Assaying Kit according to the manufacturer's instruction (Active Motif, North American). This is an ELISA based quantitative assay of NF-κB binding activity using antibody directed to NF-κB p65 subunit. Nuclear NF-κB binding activity was also determined by gel shift analysis. The oligonucleotide containing the VCAM-1 κB sequence is as follows: 5'CTGCCCTGGGTTTCCCTTGAAGGGATTTCCCTCC GCCTCTGCAACA3'. The sequences of the NF–κB consensus binding sites are underlined. The DNA binding reaction was performed at 30^{o}C for 15 min in a volume of 20 μL, containing 5 μg of nuclear extract, 225 μg/mL bovine serum albumin, 1.0x10^{5} cpm of ^{32}P-labeled probe, 0.1 μg/mL poly(dI-dC), and 15 μL of binding buffer (12 mmol/L HEPES, pH 7.9, 4 mmol/L Tris, 60 mmol/L KCl, 1 mmol/L EDTA, 12% glycerol, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride). After the binding reaction, the samples were subjected to electrophoresis in 1 x Tris-glycine

buffer using 4% native polyacrylamide gels. Autoradiography was performed with a PhosphorImager 445sI (Molecular Dynamics, Sunnyvale, CA).

Adenoviruses. The adenovirus encoding myc-tagged cDNA of dominant negative N17Rac1 (Ad.N17Rac1), and cDNA of human Cu/Zn superoxide dismutase (Ad.SOD) and human catalase (Ad.Catalase) were generous gifts of Toren Finkel (National Institute of Health) and have been previously described (Sundaresan et al., 1995; Sulciner et al., 1996b; Moldovan et al., 1999). The viruses were amplified in HEK 293 cells and purified on double cesium gradients. Infection was carried out with the indicated multiplicity of infection (MOI) for indicated times, after which the infection medium was aspirated and replaced with fresh medium. The Ad.LacZ, an adenovirus encoding the E.coli LacZ gene, was used as a control for adenovirus infection. The ability of infected HAEC to express N17Rac1 were assessed by Western blot analysis via the myc-epitope tag using mouse monoclonal anti-myc antibody 9E10, which recognizes the myc peptide only on fusion proteins and not in endogenous myc proteins (Santa Cruz BioTechnology, Inc).

Western blot analysis: HAEC were lysed for 30 min on ice in 1 ml of a lysis buffer containing 0.5 % Nonidet P-40, 50 mM Hepes (pH 7.3), 150 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Protein samples (15 μg) were subjected to electrophoresis on 10% or 15% SDS-PAGE gels, and transferred to a nitrocellulose membrane. Antibody-bound protein bands are then visualized via HRP-

dependent chemiluminescence (Amersham, Corp). Anti-SOD and anti-catalase antibodies were purchased from Calbiochem Corp. Anti-Rac1 antibodies were obtained from Upstate and anti-IκB-α antibodies were obtained from Santa Cruz BioTechnology, Inc.

Results

The dominant negative mutant N17Rac1 inhibits TNF-α induced VCAM-1, E-selectin and ICAM-1 mRNA accumulation in HAEC. We first characterized the expression of N17Rac1 in Ad.N17Rac1 infected HAEC. As shown in figure 1, HAEC were infected with either Ad.LacZ (MOI of 100) or Ad.N17Rac1 (MOI of 25, 50 and 100) for 24 hours. By Western blot analysis, myc-tagged N17Rac1 protein levels were increased in a dose dependent manner after infection with Ad.N17Rac1. HAEC that were mock infected or infected with Ad.LacZ had no myc-N17Rac1 protein expression (figure 1A). Infection of HAEC with Ad.N17Rac1 (MOI of 100) also induced a time-dependent increase in the expression of myc-tagged N17Rac1. There is a progressive increase in the levels of myc-tagged N17Rac1 from 6 to 48 hours in HAEC infected with Ad.N17Rac1.

To determine the role of Rac1 in the regulation of vascular adhesion molecule expression, HAEC were infected with Ad.N17Rac1 or Ad.LacZ at MOI of 100 for 24 or 48 hours. Then HAEC were treated with TNF-α (100 U/ml) for 4 hours. As shown in figure 2A, TNF-α induced a marked increase in VCAM-1, E-selectin and ICAM-1 mRNA levels in Ad.LacZ (MOI of 100) infected cells. Infection of HAEC with Ad.N17Rac1 at MOI of 100 for 24 hours inhibited TNF-α induced VCAM-1 and E-selectin, but not ICAM-1, mRNA levels. However, at 48 hour post Ad.N17Rac1 infection, TNF-α induced VCAM-1, E-selectin and ICAM-1 mRNA accumulation were all inhibited (figure 2B). We further explore the dose effects of Ad.N17Rac1. As shown in figure 2C, infection of HAEC with dominant negative Ad.N17Rac1 at MOI of

25, 50 and 100 for 48 hours produced a dose dependent inhibition of TNF-α induced VCAM-1 and E-selectin mRNA levels. Infection with Ad.N17Rac1 only at MOI of 100, but not 25 or 50, inhibited TNF-α induced ICAM-1 mRNA upregulation.

The dominant negative Ad.N17Rac1 inhibits TNF-α induced cell surface expression of adhesion molecules. To determine whether inhibition of endothelial cell adhesion molecule expression by Ad.N17Rac1 occurs at the cell surface level, we infected HAEC with Ad.LacZ or Ad.N17Rac1 for 24 hours at indicated MOI, and treated HAEC with TNF-α for 16 hours. As shown in figure 3A, Ad.N17Rac1 at MOI of 100 and 50 inhibited TNF-α induced VCAM-1 (upper panel) and E-selectin (middle panel) cell surface expression in a dose-dependent manner. In contrast, a 24-hour infection with Ad.N17Rac1 did not inhibit TNF-α induced ICAM-1 protein expression (lower panel). However, when HAEC were infected with Ad.N17Rac1 for 48 hours, TNF-α induced ICAM-1 protein expression was also suppressed (figure 3B, lower panel). These data suggest that Rac1 is involved in TNF-α induced VCAM-1, E-selectin and ICAM-1 gene expression. These data also demonstrate that ICAM-1 gene upregulation is relatively refractory to Rac1 inhibition compared to VCAM-1 and E-selectin.

The dominant negative Ad.N17Rac1 does not inhibit TNF-α induced NF-κB translocation and IκBα degradation. The genes for VCAM-1, ICAM-1 and E-selectin contain promoter elements with recognition sites for the transcription factor NF-κB (Collins et al., 1995). To determine if N17Rac1 inhibits VCAM-1, E-selectin and

ICAM-1 gene expression through inhibition of NF-κB activation, we examined the effect of inhibition of Rac1 on TNF-α induced NF-κB nuclear translocation through two approaches: nuclear NF-κB binding activity and IκB degradation. HMEC were infected with Ad.N17Rac1 (MOI of 100) or Ad.LacZ (MOI of 100) for 48 hours and treated for 1 hour with TNF-α (100 U/ml). Nuclear extracts were used to perform NF-κB binding analysis using TransAM NF-κB p65 Transcriptional Factor Assaying Kit. As expected, TNF-α induced nuclear NF-κB binding activity in HMEC (Figure 4A, lane 2). Infection with Ad.LacZ had no effect on basal or TNF-α induced nuclear NF-κB binding activity (lanes 3 and 4). Infection with Ad.N17Rac1 did not suppress TNF-α induced NF-κB nuclear binding activity (Figure 4A, lane 6). Similarly, infection with dominant negative N17Rac1 did not inhibit TNF-α induced NF-κB nuclear binding activity in HAEC by gel mobility shift analysis (data not shown). These data suggest that Rac1 is not involved in TNF-α induced NF−κB nuclear translocation.

A crucial regulatory control point in NF-κB activation is IκB degradation through the action of the proteasome (Baeuerle and Henkel, 1994; Senftleben and Karin, 2002). We examined the effect of dominant negative N17Rac1 on TNF-α induced degradation of IκB-α. HAEC were infected with either Ad.LacZ or Ad.N17Rac1 at MOI of 100 for 48 hours and then exposed to TNF-α for 10 min. Whole cell extracts were prepared and intracellular IκB-α levels were determined by Western blot analysis. Infection with Ad.N17Rac1 results in a significant increase in Rac1 protein levels as compared to that of Ad.LacZ infected cells (figure 4B). Treatment with TNF-α induced a decrease in IκB-

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α levels in 10 min in Ad.LacZ infected cells. Ad.N17Rac1 did not inhibit TNF-α induced degradation of IκB-α in HAEC (figure 3B). These data suggest that Rac1 is not involved in TNF-α induced IκB degradation and nuclear translocation of NF-κB in HAEC.

The dominant negative N17Rac1 suppresses TNF-α induced transactivation of the NF-κB driven HIV-κB promoter and 288 VCAM-1 promoter. To explore whether the nuclear translocated NF-κB in the presence of N17Rac1 is able to transactivate NF-κB driven promoter, we transiently transfected HMEC with p(HIVκB)$_4$-CAT, a construct consisting of 4 HIV κB-binding sites (Kunsch et al., 1992). HMEC were treated with TNF-α (100 U/ml) for 16 h. As expected TNF-α induced more than 8 fold increase in p(HIVκB)$_4$-CAT promoter activity in the presence of empty vector (Figure 5A). Expression of dominant negative N17Rac1 inhibited TNF-α-induced transactivation of p(HIVκB)$_4$-CAT promoter activity (Figure 5A). These data suggest that the dominant negative Rac1 suppresses TNF-α induced transactivation of NF-κB, but not nuclear translocation of NF-κB in endothelial cells.

To investigate whether dominant negative N17Rac1 can inhibit VCAM-1 gene transcription, p288VCAM-Luc was co-transfected with an expression vector for N17Rac1. As expected, TNF-α induced a marked increase in p288VCAM-Luc promoter activity in the presence of empty vector (Figure 5B). Expression of dominant negative Rac1 inhibited TNF-α induced transactivation of p288VCAM-Luc promoter activity

(Figure 5B). These data suggest that Rac1 suppresses TNF-α induced VCAM-1 gene transcription through inhibition of NF-κB mediated transactivation in endothelial cells.

Expression of SOD inhibits TNF-α induced VCAM-1, E-selectin, and ICAM-1 mRNA and cell surface protein expression in HAEC. Activation of Rac1 by cytokine is associated with increased production of $O_2^{-\cdot}$ in a variety of cells including endothelial cells (Sulciner et al., 1996a; Sulciner et al., 1996b). Oxidant signals play a role in the regulation of endothelial cell adhesion molecules such as VCAM-1 (Marui et al., 1993). We used an adenovirus expressing SOD to determine the role of $O_2^{-\cdot}$ in the regulation of vascular adhesion molecule expression. SOD is responsible for converting O_2^{-} to H_2O_2. HAEC were infected with Ad.SOD (MOI of 100) or Ad.LacZ (MOI of 100) for 24 hours. Then, HAEC were treated with TNF-α (100 U/ml) for 4 h. By Western blot analysis, infection of HAEC with Ad.SOD increased intracellular Cu/ZnSOD protein levels (figure 6A). By Northern analysis, TNF-α induced increases in VCAM-1, E-selectin and ICAM-1 mRNA levels were inhibited by infection with Ad.SOD in HAEC (figure 6B).

To determine whether Ad.SOD treatment inhibits cell surface expression of endothelial cell adhesion molecules, we infected HAEC with Ad.LacZ or Ad.SOD for 24 hours at MOI of 100, and treated HAEC with TNF-α for 16 hours. As shown in figure 8, Ad.SOD inhibited TNF-α induced VCAM-1 (upper panel), E-selectin (middle panel) and ICAM-1 (lower panel) protein expression at the cell surface. These data suggest that $O_2^{-\cdot}$

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is involved in TNF-α induced VCAM-1, E-selectin and ICAM-1 gene expression in endothelial cells.

Expression of catalase only partially inhibits TNF-α induced E-selectin expression, but has no effect on VCAM-1 and ICAM-1 gene expression in HAEC. $O_2^{-\cdot}$ generated in cells can be converted spontaneously or enzymatically into H_2O_2. To determine the role of H_2O_2 in the regulation of vascular adhesion molecule expression, we used an adenovirus expressing catalase, which can convert H_2O_2 into H_2O. HAEC were infected with Ad.Catalase (MOI of 100) or Ad.LacZ (MOI of 100) for 24 hours, followed by treatment with TNF-α (100 U/ml) for 4 hours. Infection of HAEC with Ad.Catalase for 24 hours results in a 4-fold increase in intracellular catalase protein levels by Western blot analysis (figure 7A). By Northern blot analysis, infection with Ad.Catalase only partially inhibited TNF-α induced E-selectin mRNA levels by approximately 50% (figure 7B, panel 1). Expression of catalase had no effect on TNF-α induced VCAM-1 and ICAM-1 gene expression.

To determine the effects of Ad.Catalase treatment on cell surface expression of endothelial cell adhesion molecules, we infected HAEC with Ad.LacZ or Ad.Catalase for 24 hours at MOI of 100, and treated HAEC with TNF-α for 16 hours. As shown in figure 8, Ad.Catalase inhibited TNF-α induced E-selectin (middle panel) by approximately 50%. In contrast, Ad.Catalase treatment had no effect on TNF-α induced and VCAM-1 (upper panel) and ICAM-1 (lower panel) protein expression at cell surface.

These data suggest that H_2O_2 is partially involved in TNF-α induced E-selectin, but not VCAM-1 and ICAM-1 gene expression.

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Discussion

Regulation of endothelial cell adhesion molecule expression, particularly VCAM-1, is mediated by redox-coupled signaling mechanisms involved in the activation of the transcription factor NF-κB (Marui et al., 1993). However, the signaling pathway and the specific reactive oxygen species that regulate the expression of these proteins are not well understood. In the present study, we demonstrated that dominant negative N17Rac1 suppresses TNF-α induced VCAM-1 and E-selectin gene expression, and to a lesser degree ICAM-1 gene expression. We further demonstrated that dominant negative N17Rac1 inhibits TNF-α induced activation of NF-κB-dependent transcriptional activity, but has no effect on TNF-α induced NF-κB nuclear translocation. Furthermore, inhibition of generation of $O_2^{-\cdot}$, a downstream signal of Rac1, by SOD suppresses TNF-α induced VCAM-1, E-selectin and ICAM-1 gene expression. Our results suggest that Rac1 and $O_2^{-\cdot}$ play critical roles in TNF-α mediated expression of endothelial cell adhesion molecules.

Rac proteins are involved in the assembly of NADPH oxidase and $O_2^{-\cdot}$ generation (Bokoch, 1994). NADPH oxidase is the major source of $O_2^{-\cdot}$ in endothelial cells (Mohazzab et al., 1994) and plays an essential role in TNF-α induced $O_2^{-\cdot}$ generation (Li et al., 2002). Rac1 is involved in the activation of NADPH oxidase in endothelial cells (Abid et al., 2001) and is required for $O_2^{-\cdot}$ generation in response to inflammatory

stimuli in a variety of cell types including endothelial cells (Yeh et al., 1999; Deshpande et al., 2000; Ozaki et al., 2000). Using an NADPH oxidase inhibitor diphenylene iodonium, we previously reported that flavin binding proteins such as NADPH oxidase, are required for TNF-α induced $O_2^{-\cdot}$ generation and the activation of VCAM-1 and ICAM-1 gene expression (Tummala et al., 2000). Consistent with our early results, our finding that SOD, but not catalase, inhibits TNF-α induced endothelial cell adhesion molecule expression provides the first direct evidence that $O_2^{-\cdot}$, but not H_2O_2, is involved in the redox-sensitive regulation of inflammatory gene expression. A mechanism by which $O_2^{-\cdot}$ may mediates endothelial cell adhesion molecule expression may occur through its downstream reactive species peroxynitrite, generated from interaction of $O_2^{-\cdot}$ with nitric oxide (Tarpey and Fridovich, 2001). Recent studies indicate that peroxynitrite may function as an intracellular signal for the production of IL-8 (Zouki et al., 2001). Exogenous peroxynitrite has been shown to stimulate NF-κB activation in endothelial cells (Cooke and Davidge, 2002) and monocytes (Matata and Galinanes, 2002). Peroxynitrite also activates expression of inducible nitric oxide synthase and IL-6 (Cooke and Davidge, 2002; Matata and Galinanes, 2002).

In the present study, we found that the inhibition of Rac1 results in differential inhibitory effects on the induction of VCAM-1, E-selectin and ICAM-1 by TNF-α. Among three adhesion molecules studied, VCAM-1 and E-selectin were more profoundly inhibited by dominant negative N17Rac1 compared to ICAM-1. We showed that ICAM-1 is more resistant to the Rac1 inhibition and can only be suppressed

by a higher Ad.N17Rac1 dose. Similar differential inhibitory effects have been observed with antioxidant treatment. We demonstrated previously that the treatment of endothelial cells with thiol antioxidants such as pyrolidine dithiocarbamate or N-acetylcysteine selectively inhibit TNF-α induced VCAM-1 and to a lesser degree E-selectin gene expression, but have no effects on ICAM-1 gene expression (Marui et al., 1993). Relative differences in the inhibitory effects of N17Rac1 may be explained by the fact that different combinations of transcriptional factors are required for the activation of E-selectin, VCAM-1 and ICAM-1 promoters (Collins et al., 1995). Although all these endothelial cell adhesion genes have NF-κB binding sites, other transcription factors have also been shown to contribute to the regulation of expression of these genes. In the case of ICAM-1, Ets-, STAT- and interferon-γ-response element-dependent transcriptional mechanisms are required for ICAM-1 gene upregulation (Duff et al., 1997; Audette et al., 2001; Roy et al., 2001).

The present study shows that SOD is more efficient in inhibition of ICAM-1 gene expression than dominant negative N17Rac1 (figure 6 vs. figure 2). These data suggest that in addition to Rac1-mediated $O_2^{-\cdot}$ generation, other cellular sources such as mitochondria (Thannickal and Fanburg, 2000) may also generate $O_2^{-\cdot}$, and contribute to ICAM-1 gene upregulation. Inhibition of Rac1 can only suppress NADPH oxidase-mediated $O_2^{-\cdot}$generation, but has no effects on mitochondria-mediated $O_2^{-\cdot}$generation. In contrast, SOD can scavenge $O_2^{-\cdot}$generated from all cellular sources and may therefore be more efficient in suppressing ICAM-1 gene expression.

Two separate signaling events are involved in the NF-κB activation pathway. First, NF-κB dimers, kept in the cytoplasm through interaction with inhibitory proteins IκB, become activated by phosphorylation and degradation of IκB resulting in the subsequent release and nuclear translocation of NF-κB. This pathway depends on the IκB kinase which is essential for inducible IκB phosphorylation and degradation (Zandi et al., 1997). The second pathway regulates the transactivating potential of the p65 subunit of NF-κB once it is bound to its consensus sequence (Bergmann et al., 1998; Jefferies and O'Neill, 2000). Several studies demonstrated that upon stimulation with either TNF-α or IL-1β, the p65 subunit of NF-κB becomes phosphorylated on multiple serine sites thus potentially acting to enhance NF-κB p65 transactivating potential (Wang and Baldwin, 1998; Wang et al., 2000). The kinase directly responsible for phosphorylation of NF-κB p65 has yet to be identified, although casein kinase II has been shown to phosphorylate transactivation region found in the COOH-terminal domain of p65 (Wang et al., 2000). Calmodulin-dependent protein kinase IV has been reported to stimulate NF-κB transactivation via phosphorylation of the p65 subunit (Jang et al., 2001).

Rac1 plays an important role in the regulation of NF-κB activation evoked by environmental stresses and proinflammatory cytokines (Sulciner et al., 1996a). In the present study, we demonstrated that dominant negative N17Rac1 inhibits TNF-α induced transactivation of NF-κB-driven promoter in HMEC, but has no effect on TNF-

α induced nuclear NF-κB translocation in both HAEC and HMEC. These data are consistent with other studies. Jefferies and co-workers reported that inhibition of Rac1 suppressed IL-1β driven p65-mediated NF-κB transactivation, but had no effect on IL-1β induced nuclear NF-κB translocation or IκBα degradation in a thymoma cell line and in porcine aortic endothelial cells (Jefferies and O'Neill, 2000). Inhibition of NF-κB transactivation by the suppression of phosphatidylinositol 3-kinase (PI3K) has also been reported. Sizemore and co-workers reported that inhibition of PI3K suppressed NF-κB-dependent gene expression, but had no effect on the IL-1β stimulated degradation IκB-α, or NF-κB nuclear translocation and DNA binding. In contrast, PI3K inhibitors blocked the IL-1β stimulated phosphorylation and transactivation of NF-κB p65 (Sizemore et al., 1999). However, other studies have found that Rac1 mediates NF-κB activation through regulation of NF-κB nuclear translocation in HeLa cells (Sulciner et al., 1996a). Sanlioglu and co-workers reported that dominant negative N17Rac1 inhibits lipopolysaccaride-induced nuclear NF-κB binding activity (Sanlioglu et al., 2001). Similarly, inhibition of Rac1 suppressed TNF-α-induced nuclear NF-κB binding activity in human umbilical vein endothelial cells (Deshpande et al., 2000). These conflicting observations on the mechanism of Rac1 in NF-κB activation illustrate the complex systems of interaction between Rac1 and NF-κB pathways as well as cell type differences in this interaction. Nevertheless, we provide evidence that Rac1 is not involved in TNF-α induced IκB-α degradation and NF-κB nuclear translocation. Instead, Rac1 plays a role in the modulation of the ability of the NF-κB to transactivate gene expression in endothelial cells.

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Our findings suggest that the activation of Rac1-dependent pathways and $O_2^{-\cdot}$ generation are required for the upregulation of endothelial adhesion molecules stimulated by TNF-α. Cumulatively, our results may provide the molecular link between Rac1, NADPH oxidase and $O_2^{-\cdot}$ in the signaling pathway that mediates TNF-α induced expression of endothelial cell adhesion molecules, suggesting that Rac1-dependent signaling pathways may serve as important pharmacological targets for the treatment of inflammatory diseases.

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Figure Legends

Figure 1. Characterization of myc-tagged N17Rac1 expression after infection with Ad.N17Rac1. A) HAEC were infected with Ad. LacZ (MOI of 100) or Ad.N17Rac1 (MOI of 25, 50 and 100) for 24 hours. **B)** HAEC were infected with Ad.N17Rac1 (MOI of 100) for 6, 24 and 48 hours. Whole cell lysates were harvested and analyzed by immunoblotting with an antibody to myc.

Figure 2. The dominant negative N17Rac1 inhibits TNF-α induced VCAM-1, E-selectin and ICAM-1 mRNA accumulation. A) HAEC were infected with Ad.LacZ (MOI of 100; lanes 1 and 2) or Ad.N17Rac1 (MOI of 100; lanes 3 and 4) for 24 hours and then exposed to TNF-α (100 U/ml) for 4 h (lanes 2 and 4). **B.** Untreated HAEC (lanes 1 and 2) or HAEC infected with Ad.LacZ (MOI of 100; lanes 3 and 4), Ad.N17Rac1 (MOI of 100; lanes 5 and 6) for 48 hours were exposed to TNF-α (100 U/ml) for 4 h (lanes 2, 4 and 6). **C)** HAEC were infected with Ad.LacZ (MOI of 100; lanes 1 and 2) or Ad.N17Rac1 (MOI of 25, 50 and 100; lanes 3, 4, 5 and 6) for 48 hours and then exposed to TNF-α (100 U/ml) for 4 h (lanes 2 and 4, 5 and 6). Total RNA was isolated and VCAM-1, E-selectin and ICAM-1 mRNA levels were determined by Northern analysis. Two independent experiments showed similar results.

Figure 3. The dominant negative N17Rac1 inhibits TNF-α induced cell surface expression of VCAM-1, E-selectin and ICAM-1. A) Untreated HAEC (lanes 1 and 2) and HAEC infected with Ad.LacZ (MOI of 100; lanes 1 and 2) or Ad.N17Rac1 (MOI of 25, 50 and 100) for 48 hours were exposed to TNF-α (100 U/ml) for 16 h. **B)** HAEC were infected with Ad.LacZ (MOI of 100) or Ad.N17Rac1 (MOI of 100) for 48 hours and then exposed to TNF-α (100 U/ml) for 16 h (lanes 2 and 4). Cell surface expression of adhesion molecules were determined by ELISA as described in Methods. Values represent mean+SD, n=4. *P<0.05 compared with TNF-α alone treated group.

Figure 4. The dominant negative N17Rac1 does not inhibit TNF-α induced nuclear NF-κB binding activity and IκB-α degradation. A) Untreated HMEC (lanes 1 and 2) or HMEC infected with Ad.LacZ (MOI of 100; lanes 3 and 4), Ad.N17Rac1 (MOI of 100; lanes 5 and 6) for 48 hours, were exposed to TNF-α (100 U/ml) for 1 hour (lanes 2, 4 and 6). Nuclear extracts were isolated and nuclear NF-κB binding activity was determined by using TransAM NF-κB p65 Transcriptional Factor Assaying Kit. Values are mean ± SD, n=3. *: P<0.05 compared to control group. **B)** HAEC infected with Ad.LacZ (MOI of 100; lanes 1 and 2), Ad.N17Rac1 (MOI of 100; lanes 3 and 4) for 48 hours were exposed to TNF-α (100 U/ml) for 1 h (lanes 2 and 4). Whole cell lysates were analyzed by immunoblotting with antibodies to Rac1 or IκB-α.

Figure 5. The dominant negative N17Rac1 inhibits TNF-α induced transactivation of the NF-κB-driven HIV$(κB)_4$ promoter and VCAM-1 promoter in HMEC. A)

HMEC cultured in 6 well plates were transfected with 1 μg pHIV(κB)$_4$-CAT plus 1 μg pEXV-N17Rac1, or empty vector pEXV. These cells were also tranfected with 0.5 μg of pRL-TK for normalization of transfection efficiency. After a 24-hour recovery, cells were exposed to TNF-α for 16 hours, cell extracts were harvested and 5 μg of protein were used for CAT assay. **B)** HMEC cultured in 6 well plates were transfected with 1 μg p288VCAM-Luc plus 1 μg of pEXV-N17Rac1, or empty vector pEXV. These cells were also transfected with 0.1 μg of pRL-TK for normalization of transfection efficiency. After a 24-hour recovery, cells were exposed to TNF-α for 16 hours, cell extracts were harvested and luciferase assays were performed. Values are mean ± SD, n=4. *P<0.05 compared to TNF-α alone group.

Figure 6. SOD suppresses TNF-α induced VCAM-1, E-selectin and ICAM-1 mRNA accumulation. **A)** Whole cell lysates from HAEC infected with Ad.SOD at MOI of 100 for 24 hours were analyzed by immunoblotting with an antibody to SOD. **B)** HAEC were infected with Ad.LacZ (MOI of 100; lanes 1 and 2) or Ad.SOD (MOI of 100; lane 3 and 4) for 24 hours and then exposed to TNF-α (100 U/ml) for 4 h (lanes 2 and 4). Total RNA was isolated and VCAM-1, E-Selectin and ICAM-1 mRNA levels were determined by Northern analysis. Two independent experiments showed similar results.

Figure 7. Catalase only partially inhibits TNF-α induced E-selectin mRNA accumulation, but has no effect on VCAM-1 or ICAN-1 gene upregulation. A)

Whole cell lysates from HAEC infected with Ad.Catalase at MOI of 100 for 24 hours were analyzed by immunoblotting with an antibody to catalase. **B)** HAEC were infected with Ad.LacZ (MOI of 100; lanes 1 and 2) or Ad.Catalase (MOI of 100; lanes 3 and 4) for 24 hours and then exposed to TNF-α (100 U/ml) for 4 h (lanes 2 and 4). Total RNA was isolated and VCAM-1, E-Selectin and ICAM-1 mRNA levels were determined by Northern analysis. Two independent experiments showed similar results.

Figure 8. Effects of Ad.SOD or Ad.Catalase on TNF-α induced cell surface expression of VCAM-1, E-selectin and ICAM-1. HAEC infected with Ad.LacZ (MOI of 100) or Ad.SOD (MOI of 100), or Ad.Catalase (MOI of 100) for 24 hours were exposed to TNF-α (100 U/ml) for 16 h. Cell surface expression of VCAM-1 (upper panel), E-selectin (middle panel) or ICAM-1 (lower panel) was determined by ELISA as described in Methods. Values represent mean±SD, n=4. *: $P<0.05$ compared with TNF-α treated cells infected with Ad.LacZ.

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Figure 1

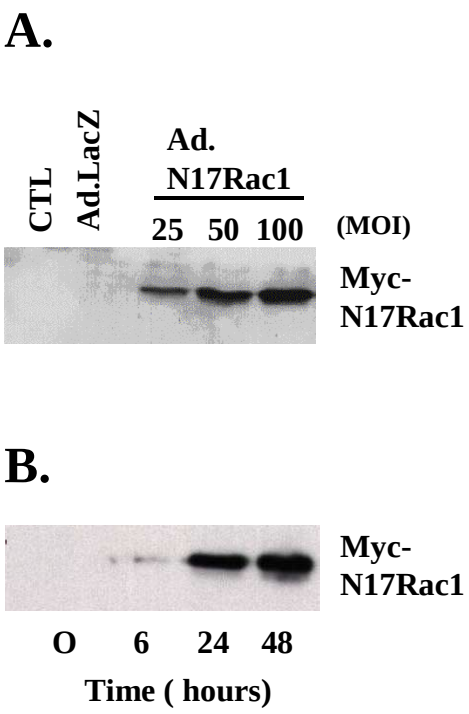

Figure 2

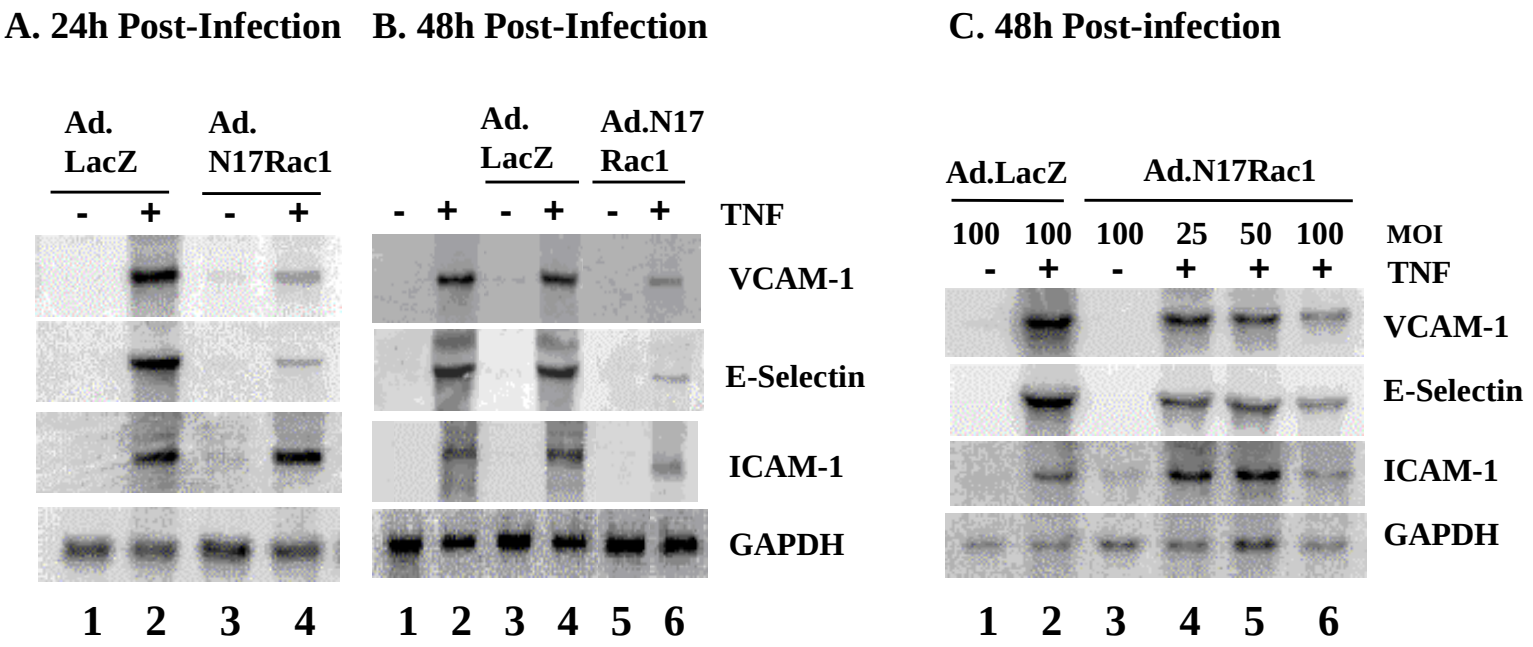

Figure 3

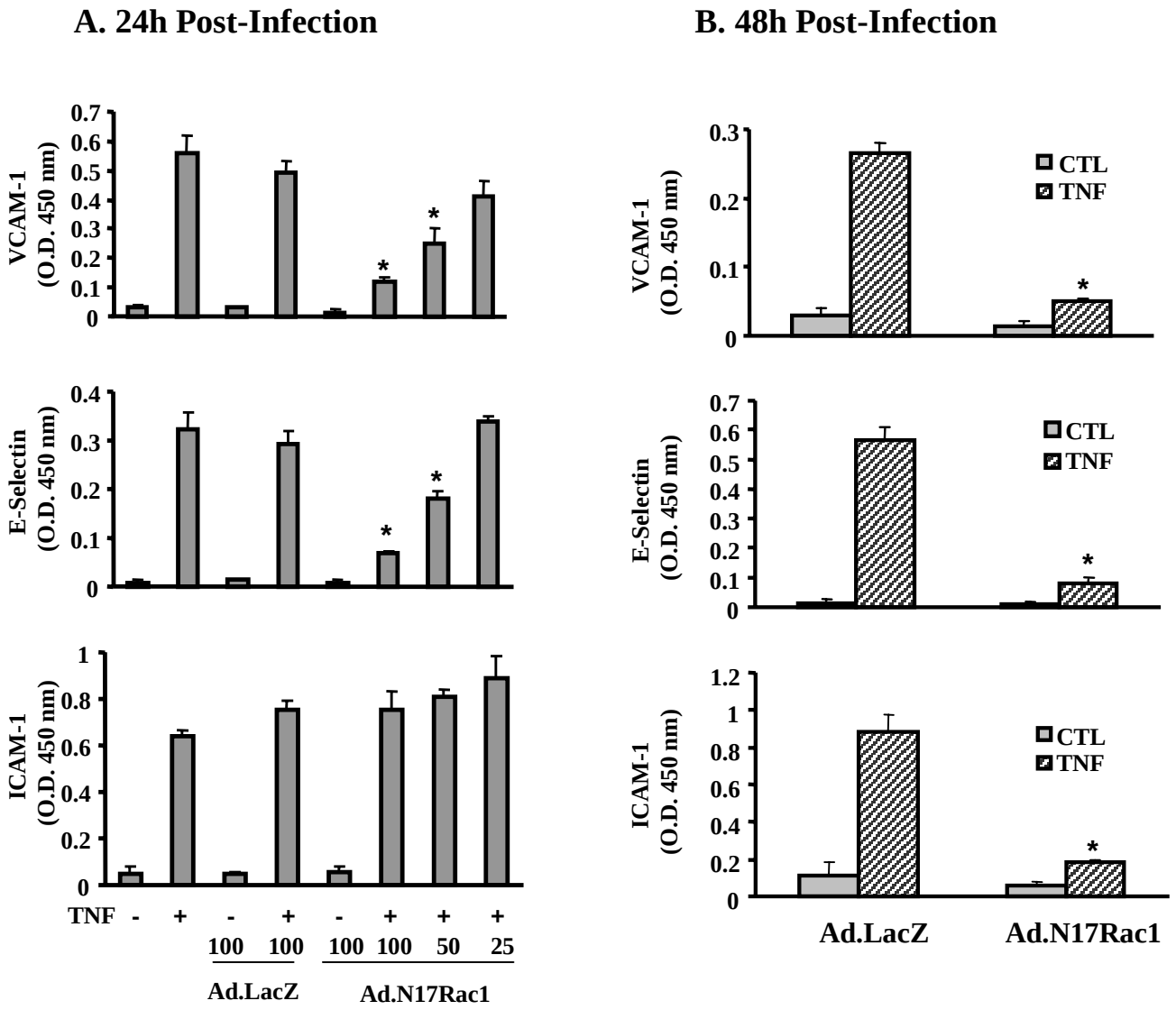

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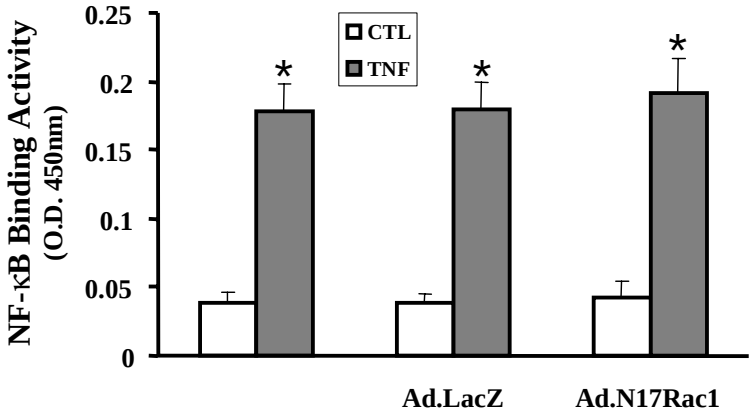
Figure 4

A. Nuclear NF-κB Binding Activity



B. IκB-α Degradation

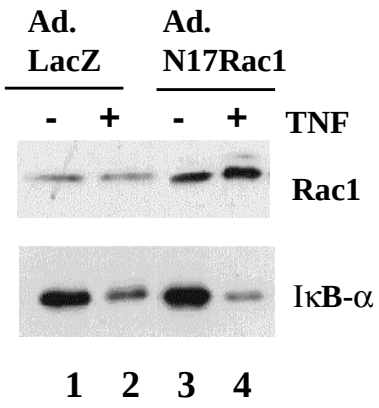


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Figure 5

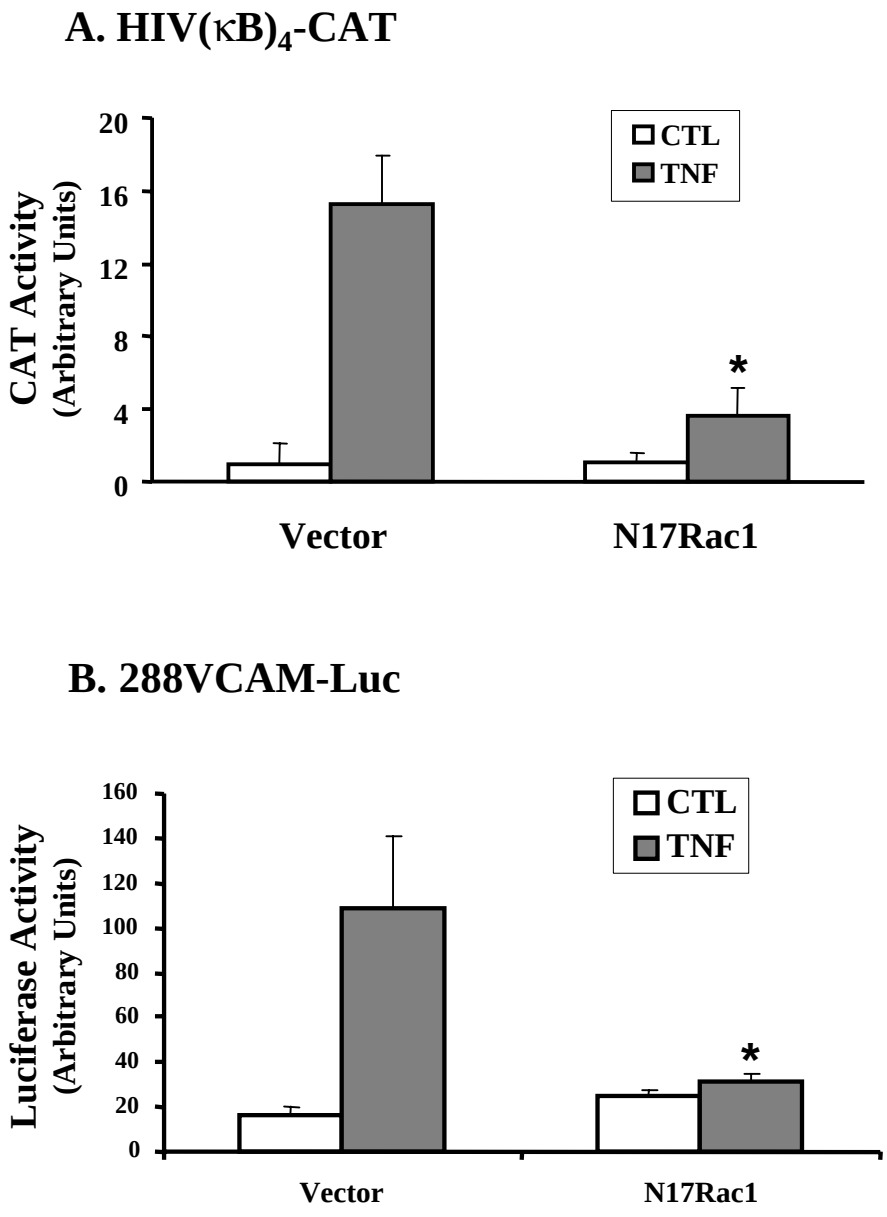

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Figure 6

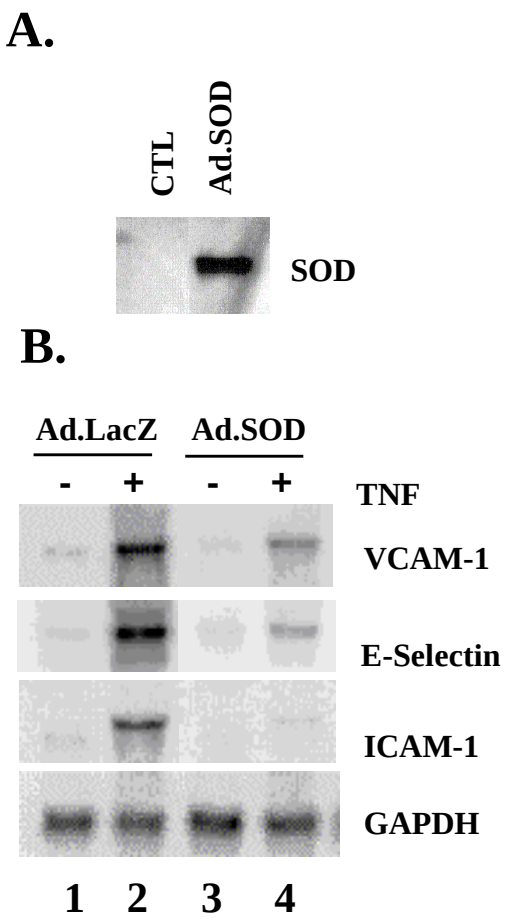

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Figure 7

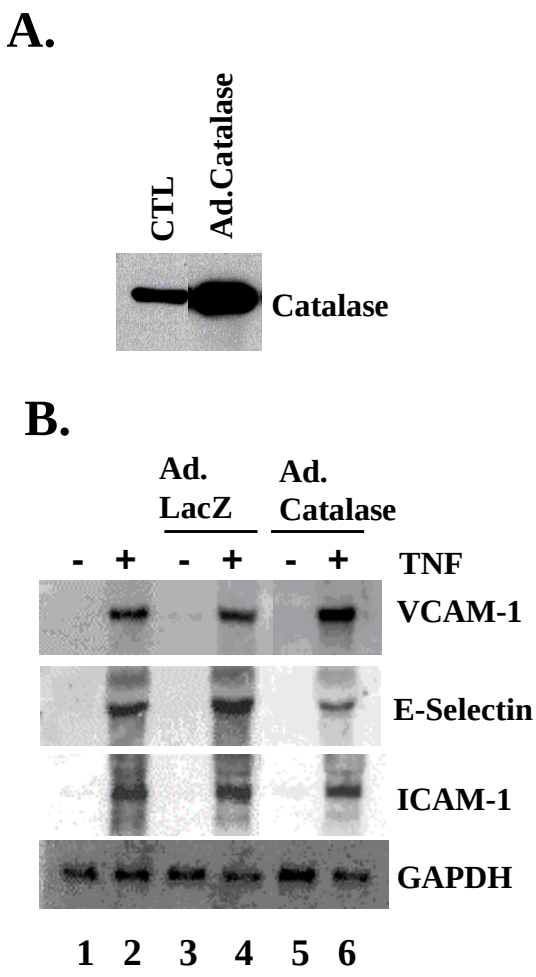

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Figure 8