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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-κB Activation in Endothelial Cells[1]

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Inflammation is a basic pathological mechanism leading to a variety of vascular diseases. The inflammatory reaction involves complex interactions between both circulating and resident leukocytes and the vascular endothelium. In this study, we report evidence for a novel action of TNF-related activation-induced cytokine (TRANCE) as an inflammatory mediator and its underlying signaling mechanism in the vascular wall. TRANCE significantly increased endothelial-leukocyte cell interactions, and this effect was associated with increased expression of the cell adhesion molecules, ICAM-1 and VCAM-1, on the endothelial cells. RT-PCR analysis and promoter assays revealed that expression of these cell adhesion molecules was transcriptionally regulated mainly by activation of the inflammatory transcription factor, NF-κB. TRANCE induced IκB-α phosphorylation and NF-κB activation via a cascade of reactions involving the TNFR-associated factors, phospholipase C, PI3K, and protein kinase C (PKC-α and PKC-ζ). It also led to the production of reactive oxygen species via PKC- and PI3K-dependent activation of NADPH oxidase in the endothelial cells, and antioxidants suppressed the responses to TRANCE. These results demonstrate that TRANCE has an inflammatory action and may play a role in the pathogenesis of inflammation-related diseases. *The Journal of Immunology,* **2005, 175: 531–540.**

Vascular inflammation is a pivotal event in the pathogenesis of many human diseases, including atherosclerosis, hypertension, restenosis, septic shock, autoimmune diseases, and ischemia/reperfusion damage (1–3). The inflammatory reaction is mediated by complex interactions between both circulating and resident leukocytes and the vascular endothelium. In health, the endothelial cell surface of the lumen is a relatively nonadhesive and nonthrombogenic conduit for the cellular and macromolecular constituents of the blood. In certain diseases, some adhesive interactions between the endothelial cells and constituents of the blood or extracellular matrix are changed by the production of adhesion molecules and their shedding onto the endothelial and leukocyte surfaces (4). These adhesion molecules are classified into two major classes: the Ig superfamily (e.g., ICAM-1 and VCAM-1) and the selectins (e.g., P-selectin and E-selectin). The activation of endothelial cells and resulting expression of adhesion molecules at sites of inflammation are of particular significance. Up-regulation of adhesion molecules on the surface of endothelial cells is prominent when they are exposed to proinflammatory molecules such as TNF-α, IL-1β, IFN-γ, platelet-derived growth factor, and vascular endothelial growth factor (VEGF)[3] (5–9). Furthermore, there is in vivo evidence of increased expression of the endothelial adhesion molecules ICAM-1 and VCAM-1 in inflammatory animal models and in human atherosclerotic plaques (10).

TNF-related activation-induced cytokine (TRANCE), also known as osteoclast differentiation factor, receptor activator of NF-κB ligand (RANKL), and osteoprotegerin ligand, is a key regulator of bone remodeling and also controls T cell/dendritic cell communication and lymph node formation (11–14). TRANCE and its decoy receptor, osteoprotegrin (OPG), are also implicated in the vascular calcification, including that of coronary and aortic vessels, which is very common and clinically significant in atherosclerosis and heart failure (15). Recently, we demonstrated a novel activity of TRANCE, namely promotion of angiogenesis in vivo, and we showed that binding of TRANCE to its receptor, receptor activator of NF-κB (RANK), stimulates the proliferation, migration, and tube formation of endothelial cells in vitro (16). This suggested that TRANCE plays a significant role in endothelial cell activation. Indeed, activation of endothelium is a pivotal step in both angiogenesis and inflammation, and is regulated positively or negatively by a variety of growth factors and cytokines. Several factors such as VEGF, platelet-derived growth factor, IL-1, and

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[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; CAM, cell adhesion molecule; CTC, chelerythrine chloride; DN, dominant negative; IKK, IκB kinase; NAC, *N*-acetylcysteine; OPG, osteoprotegrin; PKC, protein kinase C; PLC, phospholipase C; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; ROS, reactive oxygen species; TRAF, TNFR-associated factor; TRANCE, TNF-related activation-induced cytokine.

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TNF-α are well characterized for their action as an angiogenic and proinflammatory mediator (9, 17). In contrast, angiopoietin-1 and basic fibroblast growth factor are shown to promote angiogenesis, but prevent vascular inflammation (18–20).

In the present study, we confirmed that TRANCE stimulates inflammatory responses by increasing endothelial-leukocyte cell interaction and the expression of the cell adhesion molecules (CAMs), ICAM-1 and VCAM-1, on endothelial cells. These inflammatory responses occurred through a cascade of reactions involving TNFR-associated factors (TRAFs), phospholipase C (PLC), PI3K, protein kinase C (PKC-α and PKC-ζ), and reactive oxygen species (ROS), leading to NF-κB activation.

Materials and Methods

Cell culture and reagents

HUVECs were isolated from human umbilical cord veins by collagenase treatment, as described previously (21), and used in passages 2–7. They were grown in M199 medium (Invitrogen Life Technologies) supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor (Upstate Biotechnology), and 5 U/ml heparin at 37°C in a humidified 95–5% (v/v) mixture of air and CO_2. U937 cells were grown in RPMI 1640 (Invitrogen Life Technologies). Soluble TRANCE (human CD8-conjugated form) was purified from insect cells, as described previously (22).

Flow cytometry

Cells from subconfluent cultures were gently detached from wells with PBS containing 2 mmol/L EDTA, washed two or three times with PBS, and resuspended in PBS containing 2% BSA. Thereafter, they were incubated with mouse FITC-conjugated anti-human ICAM-1 and VCAM-1 Abs (Serotec) for 30 min on ice, fixed in 2% paraformaldehyde, and analyzed by flow cytometry in a FACS (BD Biosciences).

Semiquantitative RT-PCR analysis

Total RNA was obtained from HUVECs with a TRIzol reagent kit. Different amounts of total RNA (0.5–5 μg) were amplified by the RT-PCR, and the correlation between the amounts of RNA used and the levels of PCR products from both target mRNAs (ICAM-1 and VCAM-1) and the internal standard (β-actin) mRNA was examined. Briefly, target RNA was converted to cDNA by treatment with 200 U of reverse transcriptase and 500 ng of oligo(dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM dNTPs at 42°C for 1 h. The reaction was stopped by heating at 70°C for 15 min. A total of 1 μl of the cDNA mixture was then used for enzymatic amplification; PCR was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of *Taq*DNA polymerase (Promega), and 0.1 μM each of primers for ICAM-1 and VCAM-1 in a DNA thermal cycler (model PTC-200; MJ Research) under the following condition: denaturation at 94°C for 5 min for the first cycle and for 30 s starting from the second cycle, annealing at 55°C (ICAM-1) and 60°C (VCAM-1) for 30 s, and extension at 72°C for 30 s for 25 cycles. Final extension was at 72°C for 10 min. The primers used were as follows: 5′-CAGTGACCATCTACAGCTTTCCGG-3′ (sense) and 5′-GCTGCTACCACAGTGATGATGACAA-3′ (antisense) for ICAM-1; 5′-GATACAACCGTCTTGGTCAGCCC-3′ (sense) and 5′-CGCATCCTTCAACTGGCCTT-3′ (antisense) for VCAM-1.

Retroviral vectors and generation of stable transfectants

Dominant-negative TRAF2 (DN-T2) and TRAF6 (DN-T6) constructs were generously provided by Y.-Y. Kong (Pohang University, Kyungbuk, Korea). cDNA sequences encoding hemagglutinin-tagged DN-T2 and Flag-tagged DN-T6 were subcloned into pMSCVpuro vector (BD Clontech) and introduced into HEK293T cells (packaging cell line) with 1 μg of pVSV-G vector (BD Clontech) using LipofectAMINE Plus reagent, according to the manufacturer's instructions. The next day, the virus in the supernatants of these cells was added to HUVECs along with 5 μg/ml Polybrene. After 24-h incubation, the medium was removed and replaced with fresh medium containing 3 μg/ml puromycin. Puromycin-resistant clones were selected by incubating for 1 wk in the presence of 3 μg/ml puromycin. Protein expression was confirmed by Western blotting.

Plasmid construction and transient transfection

The expression vector pHACE (23) was used to generate plasmids that encode PKC mutants with a C-terminal hemagglutinin tag. pHACE-PKC-DN expression plasmids were generated by ligating full-length open reading frames of the PKC isoforms harboring a DN (K→R: PKC-α and δ or K→M: PKC-ζ) point mutation at the ATP binding site into pHACE digested with *Eco*RI. cDNA fragments corresponding to these PKC mutants were generated by PCR and confirmed by sequencing with an ABI Prism cycle sequencing kit (PerkinElmer Life Sciences). The DN PKC isoforms were introduced into HUVECs with LipofectAMINE Plus.

Reporter gene constructs and luciferase assays

To construct the ICAM-1 and VCAM-1 luciferase plasmids, we cloned regions spanning −1350 to +45 bp (full length) and −485 to +45 (truncated form) of the human ICAM-1 promoter, and −1716 to +119 bp (full length) and −213 to +119 (truncated form) of the human VCAM-1 promoter, into pGL3-basic (Promega). HUVECs were transfected with 1 μg of the plasmids and 1 μg of the control pCMV-β-gal plasmid, cell extracts were prepared 24 h after transfection, and luciferase assays were conducted with the Luciferase Assay System (Promega). Luciferase activities were normalized with parallel β-galactosidase activities to correct for differences in transfection efficiency, and the β-galactosidase assays were performed using the β-Galactosidase Enzyme Assay System (Promega).

Preparation of nuclear extracts and electrophoretic gel mobility shift assays

Cells were washed three times with ice-cold TBS and resuspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin). After 15 min, Nonidet P-40 was added to a final concentration of 0.6%, and nuclei were pelleted and suspended in 50 μl of buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin). After 30-min agitation at 4°C, the lysates were centrifuged and the supernatants were diluted with buffer C. The binding reaction involved a 30-min incubation of 15 μg of nuclear protein with a ^{32}P end-labeled, double-stranded oligonucleotide containing the NF-κB binding site of the human VCAM-1 promoter (5′-CCTTGAAGGGATTTCCCTCC-3′) (24). In Ab supershift experiments, nuclear extracts were preincubated for 30 min at room temperature with 2 μg of polyclonal rabbit Abs to human NF-κB proteins (p65, p50, p52, RelB, and c-Rel; Santa Cruz Biotechnology). The mixtures were resolved on native 5% polyacrylamide gels, which were dried and autoradiographed.

Measurement of intracellular ROS

Intracellular ROS were measured by the procedure of Koo et al. (25). Briefly, serum-starved cells on round coverslips were stabilized in serum-free medium without phenol red for at least 30 min and stimulated with TRANCE for various times. A total of 5 μM H_2DCFDA was added for the last 5 min of stimulation, and the cells were immediately observed with a laser-scanning confocal microscope (LSM410; Carl Zeiss). The samples were excited with a 488-nm Ar laser, and images were filtered with a long-pass 515-nm filter. Approximately 30 cells were selected at random in three separate experiments, and the 2′,7′-dichlorofluorescin fluorescence intensities of the treated cells were compared with those of unstimulated control cells (fold stimulation).

Adhesion assay

HUVECs were plated in 2% gelatin-coated 96-well plates at a density of 1×10^4 cells/well and stimulated with TRANCE for 16 h. Human U937 cells were then added (5×10^4 cells/ml, 200 μl/well) to the confluent HUVEC monolayers and incubated for 30 min. Thereafter, the cells in the wells were washed out three times with PBS and fixed and stained with Diff-Quick (Baxter Healthcare). Adherent cells were counted in five randomly selected optical fields of each well.

Western blotting

Cell lysates or immunoprecipitates were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blocked membranes were then incubated with the indicated Abs, and the immunoreactive bands were visualized using a chemiluminescent substrate.

Statistical analysis

The data are presented as means ± SE, and statistical comparisons between groups were performed by one-way ANOVA, followed by Student's *t* test.

Results

TRANCE induces adhesion of monocytes to HUVECs

The recruitment of leukocytes from circulating blood is crucial for the inflammatory reaction. It occurs by a multistep process in which the leukocytes interact with the endothelium of postcapillary venules (26). To determine the role of TRANCE in the inflammatory reaction, we first tested whether it induces the adhesion of leukocytes to endothelial cells. HUVECs were exposed to various concentrations of TRANCE for 16 h and cocultured with monocytic (U937) cells for an additional 30 min. As shown in Fig. 1, adhesion of the U937 cells to the HUVECs was maximally stimulated by TRANCE to an extent comparable to that achieved with 10 ng/ml VEGF.

TRANCE increases expression of ICAM-1 and VCAM-1

We next asked whether TRANCE induced the expression of ICAM-1 and VCAM-1 in the endothelial cells. TRANCE indeed increased the cellular levels of ICAM-1 and VCAM-1 (Fig. 2*B*), and increased expression of ICAM-1 and VCAM-1 was detected within 4 h of exposure to 5 μg/ml TRANCE, with a near-maximal effect at 12 h (Fig. 2, *A* and *B*). We confirmed by flow cytometry that TRANCE increased cell surface expression of these adhesion molecules on the HUVECs (Fig. 2, *C* and *D*).

TRANCE increases expression of ICAM-1 and VCAM-1 mRNAs

To determine whether TRANCE regulates endothelial ICAM-1 and VCAM-1 expression at the transcriptional level, we performed semiquantitative RT-PCR analyses and promoter assays. When HUVECs were treated with 5 μg/ml TRANCE, expression of ICAM-1 and VCAM-1 mRNAs rose within 4 h and the increase was sustained for up to 12 h (Fig. 3*A*). Pretreatment with actinomycin D, to inhibit transcription, almost completely prevented these increases (Fig. 3*B*), indicating that they resulted from increased transcription rather than stabilization of pre-existing mRNA. The human ICAM-1 promoter (1.2 kb) contains binding sites for a number of transcription factors, including NF-κB, AP-1, and STAT (27), while the human VCAM-1 promoter (1.8 kb) has binding sites for NF-κB, TRE, and GATA (28). HUVECs were transiently transfected with ICAM-1 and VCAM-1 luciferase plasmids driven by ICAM-1 and VCAM-1 promoter regions, respectively (see *Materials and Methods*). As shown in Fig. 3*C*, TRANCE in each case stimulated reporter gene activity. Although a number of *cis*-acting elements in the distal and proximal promoter regions contribute to ICAM-1 and VCAM-1 expression, the proximal NF-κB binding sites located ~200 bp (ICAM-1) and 65 and 75 bp (VCAM-1) upstream of the transcription start site have been shown to be particularly important (27, 29). To assess the role of these NF-κB elements in TRANCE-stimulated expression, we generated truncated forms of the luciferase plasmids containing only the proximal NF-κB binding sites of these promoters. As shown in Fig. 3*D*, TRANCE increased reporter gene expression from these plasmids to almost the same extent as from the original plasmids. Clearly, the NF-κB elements are important for TRANCE-induced transcription of ICAM-1 and VCAM-1 mRNAs in the endothelial cells.

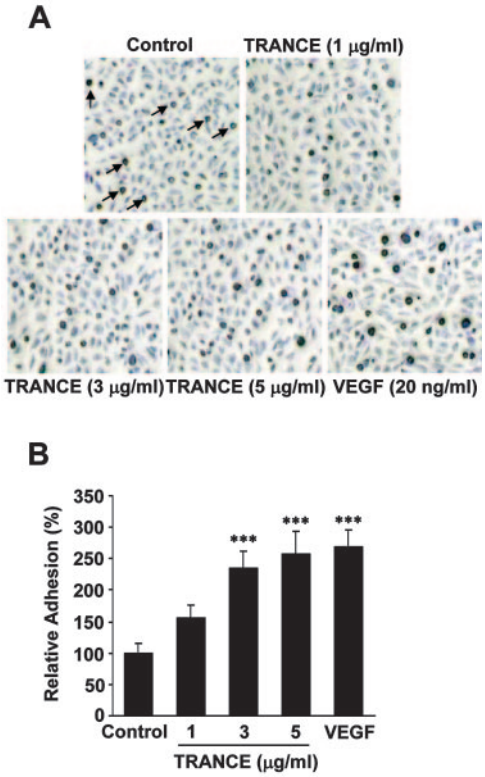

FIGURE 1. TRANCE induces adhesion of monocytes to HUVECs. *A*, HUVECs were stimulated with various concentrations of TRANCE for 15 h, or 20 ng/ml VEGF for 8 h. Adhesion to U937 human monocytes was then measured, as described in *Materials and Methods*. *B*, Data are means ± SD of relative adhesion in triplicate experiments, with control untreated cells set at 100%. ***, $p < 0.001$ vs control.

The effect of DN forms of TRAF2 and TRAF6 on TRANCE-induced ICAM-1 and VCAM-1 expression

TRANCE exerts its effects by binding to its receptor, RANK, on endothelial cells. RANK in turn interacts with members of the family of TRAFs that mediate activation of NF-κB and JNK (30–33). Among these TRAF molecules, TRAF2 and TRAF6 appear to be important components of the NF-κB signaling pathway (34). HUVECs were therefore either mock infected or infected with retroviruses encoding DN forms of TRAF2 (DN-T2) and TRAF6 (DN-T6). Overexpression of DN-T2 and DN-T6 resulted in substantial inhibition of ICAM-1 and VCAM-1 expression at both the mRNA and protein levels (Fig. 4, *B* and *C*). Consistent with these inhibitory effects, overexpression of DN-T2 and DN-T6 reduced the binding of U937 cells to the HUVECs in response to TRANCE (Fig. 4, *D* and *E*). These results indicate that TRAF2 and TRAF6 are crucially involved in TRANCE-induced leukocyte-endothelial cell interactions and expression of ICAM-1 and VCAM-1 in endothelial cells.

The downstream signaling pathway of TRAF2 and TRAF6

Recruitment of TRAF adapter proteins to the cytoplasmic domains of RANK can lead to the activation of several intracellular signaling molecules. We treated HUVECs with TRANCE in the presence or absence of various signaling inhibitors and examined expression of ICAM-1 and VCAM-1 by Western blotting and semiquantitative RT-PCR. As shown in Fig. 5, *A* and *C*, a PLC inhibitor (U73122), a PKC inhibitor (chelerythrine chloride (CTC)), and a Ca^{2+} chelator (BAPTA-AM) each suppressed TRANCE-induced expression of ICAM-1 and VCAM-1 at both the protein and mRNA levels. In addition, the effect of TRANCE was substantially inhibited by PI3K inhibitors (LY294002 or wortmannin), whereas a MEK 1/2 inhibitor (PD98059) enhanced TRANCE-induced expression. Inhibitors alone had no significant effect on the basal expression of ICAM-1 and VCAM-1 (data not shown). These results point to a positive regulatory role of PLC, PI3K, PKC, and Ca^{2+} signals in TRANCE-induced expression of endothelial adhesion molecules and a negative role of ERK.

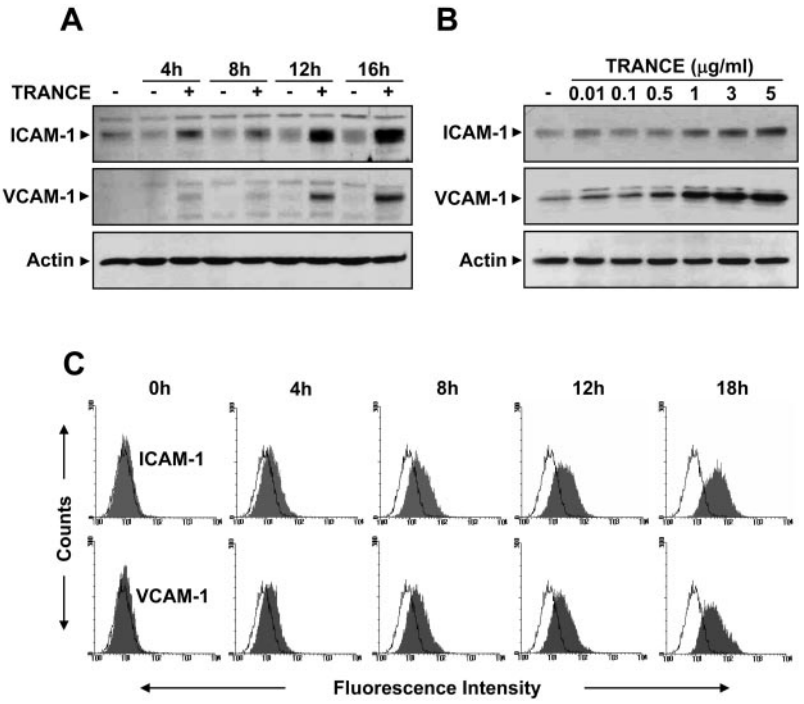

FIGURE 2. TRANCE increases expression of ICAM-1 and VCAM-1 in HUVECs. HUVECs were stimulated with 5 μg/ml TRANCE for the indicated times (*A* and *C*) or stimulated with various concentrations of TRANCE for 12 h (*B*). *A* and *B*, Western blots were probed with anti-ICAM-1 and anti-VCAM-1 Abs, and reprobed with an anti-actin Ab to verify equal loading of protein. *C*, Cell surface expression of ICAM-1 and VCAM-1 in response to TRANCE. FACScan analysis was performed with FITC-conjugated ICAM-1 and VCAM-1 Abs, as described in *Materials and Methods*.

Many studies have shown that specific isoforms of PKC integrate complex networks of signaling pathways that control the expression of adhesion molecules (35–39). PKC isoforms identified in human endothelial cells include PKC-α, PKC-δ, PKC-ϵ, and PKC-ζ (40). PKC-α is of the Ca^{2+}-dependent group, and PKC-ϵ and PKC-ζ are of the Ca^{2+}-independent group. PKC-ζ is stimulated by PI3K lipid products (41). We transfected HUVECs with DN mutants of isoforms α, δ, and ζ that abolish kinase activity, and their expression was confirmed by Western blotting (data not shown). Overexpression of the DN mutants of PKC-α and PKC-ζ reduced both ICAM-1 and VCAM-1 expression in response to TRANCE, whereas overexpression of the DN PKC-δ had no significant effect (Fig. 5, *B* and *D*). Overall, these results suggest that TRANCE-stimulated expression of ICAM-1 and VCAM-1 is mediated by activation of PLC and PI3K-dependent PKC-α and PKC-ζ.

TRANCE induces IκB-α phosphorylation

The NF-κB family of transcription factors plays a crucial role in immune, inflammatory, and apoptotic responses (42). Our data (Fig. 3*D*) suggest that activation of the NF-κB element within the ICAM-1 and VCAM-1 promoters is mainly responsible for

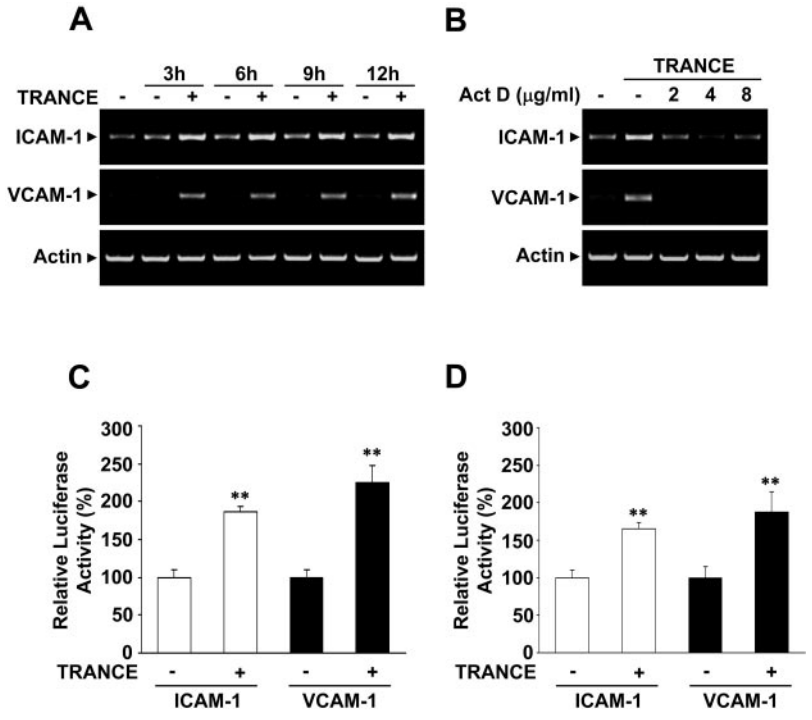

FIGURE 3. TRANCE increases transcription of ICAM-1 and VCAM-1 mRNAs by activating NF-κB in endothelial cells. *A*, HUVECs were stimulated with 5 μg/ml TRANCE for the indicated times. *B*, The cells were pretreated for 30 min with various concentrations of actinomycin D (Act D) before exposure to TRANCE (5 μg/ml). *A* and *B*, Total mRNAs were isolated, and semiquantitative RT-PCR was performed using specific primers for human ICAM-1 and VCAM-1, as described in *Materials and Methods*. Actin served as an internal control. *C* and *D*, HUVECs were transiently transfected with ICAM-1 or VCAM-1 luciferase plasmids that contain ICAM-1 (1.2 kbp) or VCAM-1 (1.8 kbp) promoter regions, respectively (*C*), or the NF-κB binding site located ~200 bp (ICAM-1) and 65 and 75 bp (VCAM-1) upstream of the transcription start site, respectively (*D*), together with a β-galactosidase plasmid, and exposed to TRANCE (5 μg/ml) for 24 h. Luciferase activity was normalized to β-galactosidase activity. Data are means ± SD of luciferase light units from triplicate experiments with the activity of untreated cells set at 100%. **, $p < 0.01$ vs − (untreated).

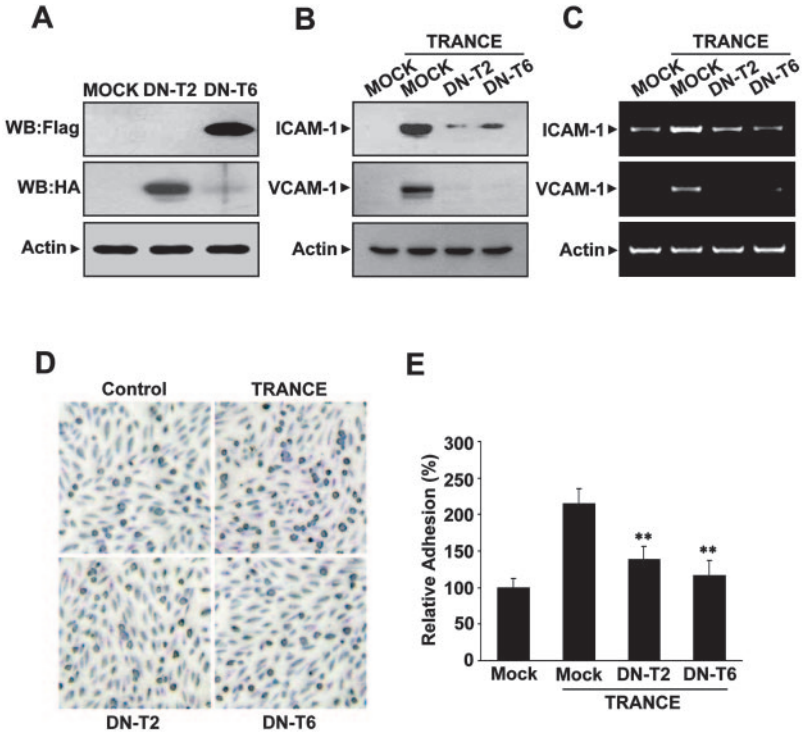

FIGURE 4. The effect of DN forms of TRAF2 and TRAF6 on TRANCE-induced ICAM-1 and VCAM-1 expression. *A*, HUVECs were stably transfected with an HA-tagged DN form of TRAF2 (DN-T2) and a Flag-tagged DN form of TRAF6 (DN-T6), as described in *Materials and Methods*, and the expression of HA-DN-T2 and Flag-DN-T6 was determined by Western blotting with anti-HA and anti-Flag Abs. The cells were stimulated with TRANCE (5 μg/ml) for 12 h (*B*) or 6 h (*C*). *B*, Western blots were probed with anti-ICAM-1 and anti-VCAM-1 Abs and reprobed with anti-actin Ab to verify equal loading of protein. *C*, Total mRNAs were isolated, and semiquantitative RT-PCR was performed using specific primers for human ICAM-1 and VCAM-1, as described in *Materials and Methods*. Actin served as internal control. *D*, HUVECs were stably transfected with DN forms of TRAF2 (DN-T2) and TRAF6 (DN-T6), and adhesion to U937 human monocytes was measured, as described in *Materials and Methods*. *E*, Data are means ± SD of relative adhesion in triplicate experiments, with control untreated cells set at 100%. **, $p < 0.01$ vs TRANCE.

TRANCE-induced transcription of ICAM-1 and VCAM-1. The activated form of NF-κB is a heterodimer generally consisting of two proteins, a p65 (also called relA) subunit and a p50 subunit (43). In the inactive state, NF-κB is found in the cytoplasm bound to IκB-α, which prevents it from entering the nucleus (44). In response to stimulation, mainly by proinflammatory cytokines, a multisubunit protein kinase, the IκB kinase (IKK), is rapidly activated and phosphorylates two critical serines in the N-terminal regulatory domain of the IκBs (45). The phoshorylated IκBs are recognized by a specific E3 ubiquitin ligase complex and degraded, so allowing nuclear translocation of the NF-κB complexes. Therefore, we examined the effect of TRANCE on IκB-α phosphorylation and degradation, by Western blotting with Abs against phospho-specific IκB-α (Ser^{32}) and IκB-α. As shown in Fig. 6*A*, TRANCE caused phosphorylation of IκB-α with maximal effect after 10 min of TRANCE stimulation and a slow decline thereafter. Some degradation of IκB-α was observed by 20 min.

We further examined the downstream signaling pathway of TRANCE. As in the case of ICAM-1 and VCAM-1 expression (Figs. 4 and 5), overexpression of DN-T2 and DN-T6 significantly reduced IκB-α phosphorylation in response to TRANCE (Fig. 6*B*), and inhibitors of PLC, PI3K, and PKC, and a Ca^{2+} chelator

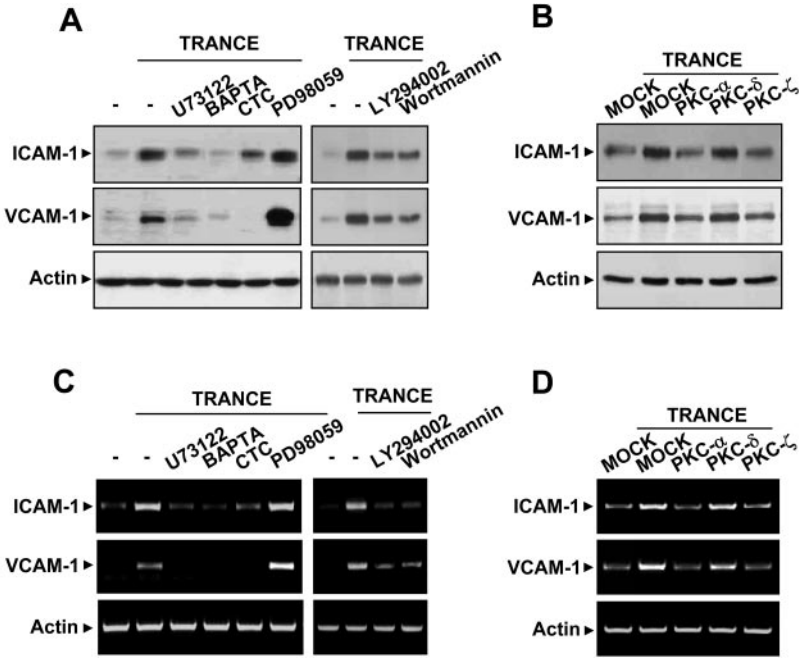

FIGURE 5. The downstream signaling pathway of TRAF2 and TRAF6 in TRANCE-induced ICAM-1 and VCAM-1 expression. HUVECs were incubated for 30 min with or without 5 μM U73122, 10 μM BAPTA-AM, 2 μM CTC, 10 μM PD98059, 10 μM LY294002, or 100 nM wortmannin before stimulation with TRANCE (5 μg/ml) for 12 h (*A*) or 6 h (*C*). The cells were then transiently transfected with expression plasmids encoding DN PKC isoforms (α, δ, ζ), as described in *Materials and Methods* (*B* and *D*). *A* and *B*, Western blots were probed with anti-ICAM-1 and anti-VCAM-1 Abs, and reprobed with anti-actin Ab to verify equal loading of protein. *C* and *D*, Total mRNAs were isolated, and semiquantitative RT-PCR was performed using specific primers for human ICAM-1 and VCAM-1, as described in *Materials and Methods*. Actin served as internal control.

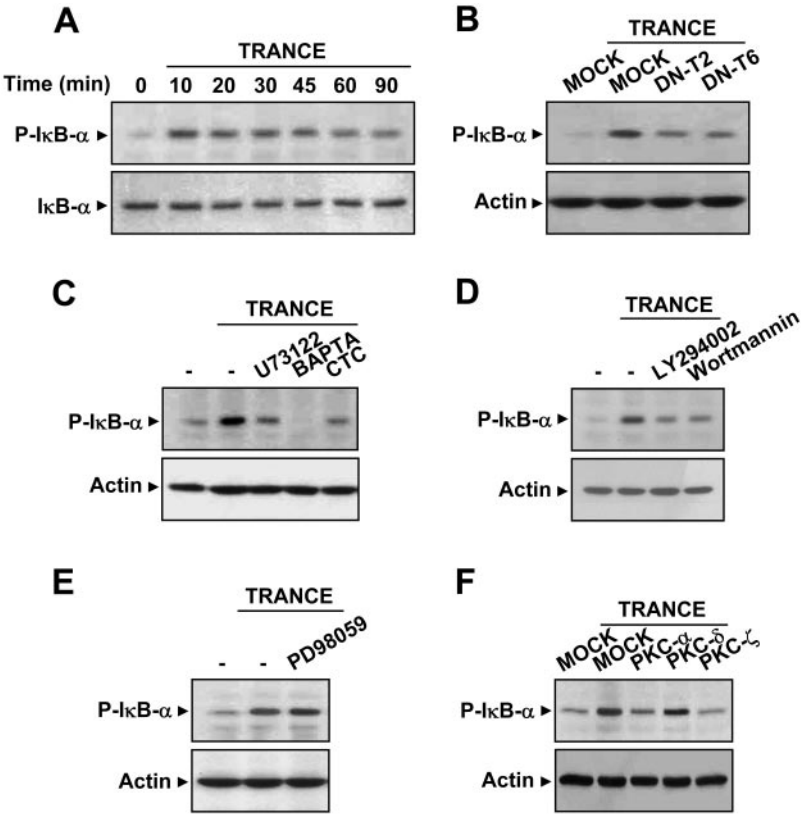

FIGURE 6. TRANCE induces IκB-α phosphorylation via the TRAF, PLC and PI3K, and PKC signaling pathway. *A*, HUVECs were stimulated with TRANCE (5 μg/ml) for the indicated times, and the phosphorylated forms of IκB-α in whole cell extracts were detected with an anti-phospho-IκB-α Ab. The membranes were then stripped and reprobed with Ab against IκB-α. HUVECs were stably transfected with an HA-tagged DN form of TRAF2 (DN-T2) and a Flag-tagged DN form of TRAF6 (DN-T6), as described in *Materials and Methods* (*B*), or incubated for 30 min with or without 5 μM U73122, 10 μM BAPTA-AM, 2 μM CTC, 10 μM LY294002, 100 nM wortmannin, or 10 μM PD98059 before stimulation with TRANCE (5 μg/ml) for 10 min (*C–E*). HUVECs were transiently transfected with expression plasmids encoding DN PKC isoforms (α, δ, ζ), as described in *Materials and Methods* (*F*). *B–F*, Phosphorylated forms of IκB-α were detected in whole cell extracts with an anti-phospho-IκB-α Ab. The membranes were stripped and reprobed with anti-actin Ab to verify equal loading of protein.

inhibited TRANCE-induced phosphorylation of IκB-α (Fig. 6, *C–E*). Similarly, expression of the DN mutants of PKC-α and PKC-ζ resulted in substantial inhibition of IκB-α phosphorylation, while the DN form of PKC-δ had no significant effect (Fig. 6*F*).

The NF-κB subunits and signaling molecules involved in TRANCE-induced NF-κB activation

We examined the effect of TRANCE on NF-κB DNA-binding activity in EMSAs with a ^{32}P end-labeled, double-stranded oligonucleotide containing the NF-κB binding site of the human VCAM-1 promoter. TRANCE increased NF-κB DNA-binding activity after 0.5 h and, although this effect declined thereafter, activity continued to be elevated up to 2 h (Fig. 7*A*). We also performed supershift experiments with Abs to p65 (RelA), RelB, c-Rel, p50, and p52. Incubation with Abs to p65 or p50, but not to RelB, c-Rel, or p52, shifted the protein-DNA complexes, and the specificity of the NF-κB DNA-binding assay was confirmed by addition of a 20-fold molar excess of unlabeled competitor

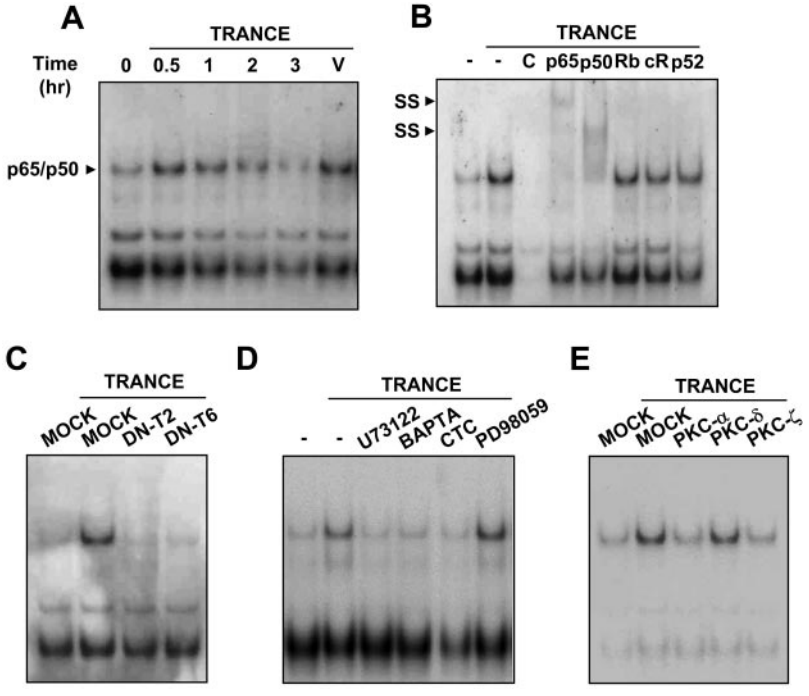

FIGURE 7. Effects of inhibitors of TRAFs, PLC, PKC, and Ca^{2+} on TRANCE-induced NF-κB DNA-binding activity. *A*, HUVECs were stimulated with TRANCE (5 μg/ml) or VEGF (20 ng/ml) for the indicated times, and nuclear extracts were incubated with a radiolabeled human VCAM-1 NF-κB oligonucleotide. *B*, Nuclear extracts from HUVECs treated with TRANCE (5 μg/ml) for 30 min were incubated in the presence or absence of a 20-fold molar excess of cold human VCAM-1 NF-κB oligonucleotide (*lane C*) or with Abs specific for p65, p50, RelB (Rb), c-Rel (cR), or p52 before adding radiolabeled human VCAM-1 NF-κB oligonucleotide. p65/p50, p65 · p50 heterodimer; SS, supershift band. *C–E*, HUVECs were stimulated with TRANCE (5 μg/ml) for 30 min, as described in Fig. 6, *B–E*. NF-κB-binding activity in nuclear extracts was measured by gel shift assay.

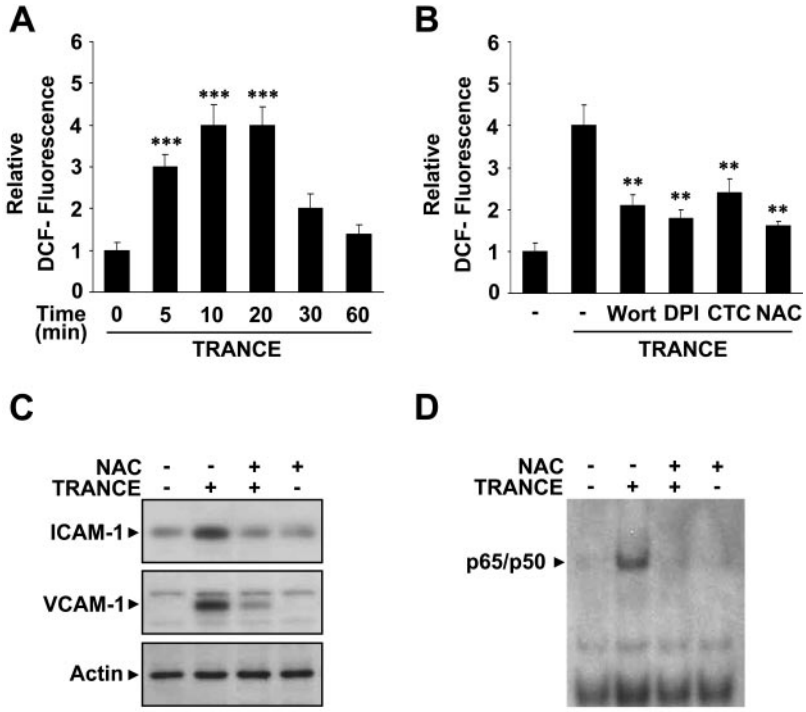

FIGURE 8. ROS are involved in TRANCE-induced NF-κB activation and CAM expression. HUVECs were stimulated with TRANCE (5 μg/ml) for the indicated times. *A* and *B*, The cells were labeled with 5 μM H_2DCFDA for the last 5 min and observed by confocal microscope, as described in *Materials and Methods*. *B*, HUVECs were preincubated for 30 min or 2 h (NAC) with or without 100 nM wortmannin, 10 μM DPI, 2 μM CTC, and 1 mM NAC before stimulation with TRANCE (5 μg/ml) for 10 min. *C* and *D*, HUVECs were preincubated for 2 h with or without 1 mM *N*-acetylcysteine before stimulation with TRANCE (5 μg/ml) for 12 h (*C*) or 30 min (*D*). *C*, Western blots were probed with anti-ICAM-1 and anti-VCAM-1 Abs. *D*, NF-κB-binding activities in nuclear extracts were measured by gel shift assay. ***, $p < 0.001$ vs 0 time; **, $p < 0.01$ vs TRANCE alone.

(Fig. 7*B*). These data indicate that TRANCE-activated NF-κB is a p65-p50 heterodimer.

To confirm the involvement of the signaling components downstream of the TRANCE receptor, we transfected HUVECs with the DN mutants of these components or treated them with various signal blockers. Overexpression of DN-T2 and DN-T6 again inhibited NF-κB DNA-binding activity in response to TRANCE (Fig. 7*C*), and the effect of TRANCE was reduced by the inhibitors of PLC and PKC and by the intracellular Ca^{2+} chelator, but slightly enhanced by the MEK 1/2 inhibitor (Fig. 7*D*). Involvement of the PKC-α and PKC-ζ isoforms, but not PKC-δ, also paralleled their roles in TRANCE-induced IκB-α phosphorylation (Fig. 7*E*).

ROS are involved in TRANCE-induced NF-κB activation and CAM expression

NF-κB is among the most important transcription factors shown to respond directly to oxidative stress in certain cell types (46). PKC has been reported to stimulate ROS production by activating NADPH oxidase (47). We therefore examined the role of ROS in TRANCE-induced NF-κB activation and CAM expression. HUVECs were treated with 5 μg/ml TRANCE for the indicated times, and ROS was detected by confocal microscopy. As shown in Fig. 8*A*, TRANCE caused a transient increase in intracellular ROS, with a maximum at 10–20 min, and this effect was reduced by pretreatment with the PI3K and PKC inhibitors (Fig. 8*B*), indicating that PKCs mediate the TRANCE-induced ROS generation. In addition, the effect of TRANCE on ROS generation was significantly reduced by the NADPH oxidase inhibitor, diphenyleneiodinium (Fig. 8*B*). Moreover, treatment of the HUVECs with the antioxidant *N*-acetylcysteine suppressed TRANCE-induced NF-κB activation and the expression of ICAM-1 and VCAM-1 (Fig. 8, *C* and *D*). Similar results were obtained with the NF-κB inhibitor pyrrolidine dithiocarbamate (data not shown). These data demonstrate that TRANCE activates NF-κB and induces CAM expression by a ROS-dependent mechanism.

Discussion

We have demonstrated a novel inflammatory action of TRANCE, and its underlying signaling mechanism, in endothelial cells. Extravasation of leukocytes from the microvasculature at sites of inflammation or injury is required for the inflammatory process (48). The endothelium is a focus for this event, which involves expression of CAMs and adhesion of leukocytes when the endothelium is activated in response to proinflammatory signals (48). We showed that TRANCE promoted endothelial-leukocyte cell interaction and that this effect was correlated with increased expression of the CAMs, ICAM-1 and VCAM-1, in the endothelial cells. We also demonstrated an important role of the inflammatory transcription factor NF-κB in the TRANCE-induced transcription of these adhesion molecules. Furthermore, TRANCE was found to induce endothelial NF-κB activation via the TRAFs/PLC and PI3K-dependent PKC and ROS signaling cascades triggered upon receptor engagement. Overall, these results suggest that TRANCE promotes vascular inflammation by activating endothelial NF-κB, which in turn enhances the expression of CAMs.

TRANCE exists either in a cell-bound form or as a truncated ectodomain variant derived by enzymatic cleavage of the cellular form by a protease of the TNF-α-converting enzyme-like family (49). TRANCE is produced mainly by osteoblast lineage cells, bone marrow stromal cells, and activated T cells (50–54), and its expression is modulated by various cytokines (IL-1, IL-6, IL-11, TNF-α), glucocorticoids, 1,25-dihydroxyvitamin D_3, dexamethasone, prostaglandin E_2, and parathyroid hormone (11, 51, 55–57). As is evident from the phenotype of RANKL-deficient mice, the biological function of TRANCE is mainly thought to be that of promoting osteoclast formation, fusion, differentiation, activation, and survival, leading to enhanced bone resorption and bone loss (58, 59). In addition, a number of studies have highlighted the involvement of TRANCE and its decoy receptor, OPG, in immune responses (60).

There is growing interest in the physiological and pathological relevance of TRANCE in the vascular system, although no vascular phenotype has yet been noted in TRANCE-deficient mice. However, there have been many recent reports of prominent expression of TRANCE in vascular cells in vitro and in vivo. Thus, primary human microvascular endothelial cells expressed both RANKL and OPG in response to the proinflammatory cytokine, TNF, although with different temporal profiles (61), and TGF-β up-regulated the expression of TRANCE mRNA and protein in bone marrow-derived endothelial cells and in primary vascular endothelial cells (62). In vivo, TRANCE protein expression increases on blood vessels and capillaries in the vicinity of resorbing osteoclasts, and in regions of active bone remodeling within sections of human osteoporotic bone (61). Moreover, this protein was also detected in small blood vessels of the skin and in arterial smooth muscle cells (63, 64). RANK is also expressed in endothelial cells of the rat coronary artery and developing blood vessels of the rat embryo in vivo, as well as in freshly isolated HUVECs (64, 65). In agreement with these patterns of expression of TRANCE and RANK in vascular cells, we recently demonstrated direct vascular effects of TRANCE and its receptor, RANK; TRANCE induced angiogenesis in vitro and in vivo by activating its receptor on endothelial cells (16). It has also been reported that VEGF up-regulates expression of RANK and increases the angiogenic response of endothelial cells to TRANCE (65), and that TRANCE regulates HUVEC survival via the PI3K/Akt signal transduction pathway (64). Thus, the TRANCE-RANK system has emerged as an important regulator of vascular function.

Although evidence for a direct vascular effect of TRANCE in inflammation-related diseases has been elusive, differential expression of TRANCE, RANK, and OPG in normal and pathological vasculature has suggested the potential role of TRANCE in the progression of atherosclerosis. Unlikely to OPG unchanged or declined, TRANCE may become up-regulated and expressed together with OPG in atherosclerotic lesions (66–68). Moreover, an increased TRANCE/OPG ratio seems consistent with the inflammatory nature of atherosclerosis (68). Mouse genetic analysis and in vitro studies have demonstrated an important role of OPG in vascular calcification, a common feature of atherosclerosis. Because mice deficient in OPG exhibit arterial calcification in addition to early onset osteoporosis (both of which are prevented by transgenic OPG expression) (69), OPG is thought to function as an important physiological suppressor of vascular calcification by counteracting the effect of TRANCE. In view of the fact that atherosclerosis is an inflammatory vascular disease that requires complex interactions between leukocytes and endothelium, TRANCE expression in the vascular region may contribute to the progression of atherosclerosis by activating endothelial cell and promoting CAM expression and extravasation of leukocytes, in addition to its role in vascular calcification.

Our data demonstrate that the TRANCE signaling pathway is involved in endothelial CAM expression (Fig. 9). Previous studies have demonstrated a role of NF-κB in the expression of both ICAM-1 and VCAM-1 in response to VEGF and thrombin, etc. (24, 29). By means of promoter assays, we demonstrated that the NF-κB element in the promoters of ICAM-1 and VCAM-1 was also crucial for TRANCE-induced CAM expression in HUVECs, and we confirmed NF-κB activation by TRANCE by measuring IκB-α phosphorylation and levels of NF-κB DNA-binding activity (Figs. 6 and 7). Many studies in other systems have demonstrated that TRAF adaptor proteins can associate with the cytoplasmic tail of RANK and lead to NF-κB activation. Wong et al. (33) first demonstrated the involvement of TRAF2, TRAF5, and TRAF6 in RANK-mediated NF-κB activation. Moreover, the central and N-terminal regions of the cytoplasmic domain of RANK that interact with TRAF6 appear to be the most crucial receptors for RANK-mediated NF-κB activation, although the C terminus of RANK that interacts with TRAF2 and TRAF5 can activate NF-κB, albeit weakly (33, 70). We showed that DN forms of TRAF2 and TRAF6 substantially inhibited TRANCE-mediated NF-κB activation (Fig. 7). This is consistent with data showing that TRAF2 and TRAF6 are essential for TRANCE-induced leukocyte-endothelial cell interactions and the expression of ICAM-1 and VCAM-1 (Fig. 4). Therefore, these results point to the importance of TRAF2 and TRAF6 in RANK-mediated inflammatory signaling in endothelial cells.

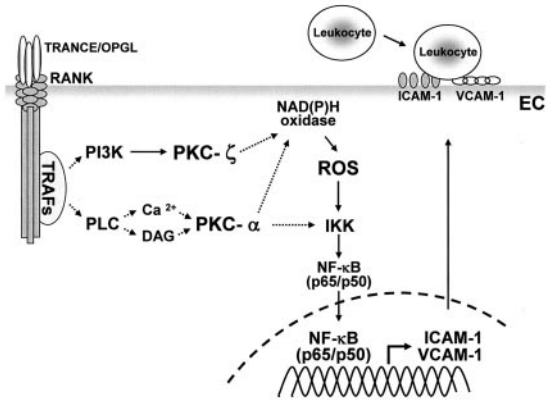

FIGURE 9. Diagram of the TRANCE signaling pathway linked to CAM expression in endothelial cells.

We have shown that PLC, PI3K, and PKC are crucial downstream signals of TRAFs leading to NF-κB activation. In a previous report, we demonstrated that TRANCE induces intracellular Ca^{2+} mobilization in HUVECs by activating PLC (16). Interestingly, the TRANCE-induced increase in intracellular Ca^{2+} was almost completely blocked by a DN form of TRAF6 and also reduced by DN forms of TRAF2 (data not shown), suggesting linkage of the TRAFs to PLC activation. PLC generates inositol 1,4,5-triphosphate and diacylglycerol, which activate intracellular Ca^{2+} mobilization and PKC, respectively. Consistent with the role of TRAFs in RANK-mediated NF-κB signaling, a PLC inhibitor (U73122), a PKC inhibitor (CTC), and a Ca^{2+} chelator (BAPTA-AM) each inhibited TRANCE-induced NF-κB activation as well as expression of ICAM-1 and VCAM-1 (Fig. 7). These results are in agreement with a recent report that TRANCE acts through PLC to release Ca^{2+} from intracellular stores, so accelerating nuclear translocation of NF-κB in osteoclasts (71).

In mammals, the PKC family consists of at least 10 isoenzymes with a broad range of tissue distributions and different cellular locations (72). Four PKC isoforms, namely PKC-α, PKC-δ, PKC-ϵ, and PKC-ζ, have been identified in human endothelial cells (40). Although the present study did not evaluate the roles of all the different PKC isoforms in TRANCE-induced NF-κB activation, we showed that at least two isoforms, PKC-α and PKC-ζ, play an important role in NF-κB activation (Fig. 7). Many PKC isoforms are critically involved in NF-κB activation in response to extracellular stimuli, via activation of IKKβ, phosphorylation of the RelA subunit of NF-κB, and ROS generation (73–75). Because DN forms of PKC-α and PKC-ζ reduced IκB-α phosphorylation in response to TRANCE, these isoforms seem to be important intermediaries in the activation of IKKβ. We also showed that TRANCE stimulates ROS generation in endothelial cells. Consistent with previous evidence that high glucose levels stimulate ROS production via PKC-dependent activation of NADPH oxidases in

vascular cells (76), the effect of TRANCE on ROS generation was inhibited by both a PKC inhibitor and an NADPH oxidase inhibitor (Fig. 8). The importance of this ROS production in NF-κB activation is demonstrated by the observation that antioxidants blocked TRANCE-induced IκB-α phosphorylation and NF-κB activation. Therefore, our data, together with the evidence that ROS lies downstream of PKC in the NF-κB pathway (37), suggest that TRANCE induces NF-κB activation at least in part via the PKC-dependent ROS-IKK pathway. The overall pathway by which TRANCE induces vascular inflammation is illustrated in Fig. 9.

In summary, the present study demonstrates that TRANCE is a vascular inflammatory molecule that increases expression of inflammatory CAMs in endothelial cells via sequential activation of PKC-α, ζ, and NF-κB-dependent signaling pathways. Our findings, together with previous observations, suggest that elevated plasma TRANCE levels are involved in causing endothelial dysfunction and atherosclerosis by increasing the transendothelial migration of leukocytes, angiogenesis, and vascular calcification.

Disclosures

The authors have no financial conflict of interest.

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