Antioxidants Inhibit Monocyte Adhesion by Suppressing Nuclear Factor-κB Mobilization and Induction of Vascular Cell Adhesion Molecule-1 in Endothelial Cells Stimulated to Generate Radicals


Abstract Cell adhesion to endothelial cells stimulated by tumor necrosis factor-α (TNF) is due to induction of surface receptors, such as vascular cell adhesion molecule-1 (VCAM-1). The antioxidant pyrrolidine dithiocarbamate (PDTC) specifically inhibits activation of nuclear factor-κB (NF-κB). Since κB motifs are present in VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) promoters, we used PDTC to study the regulatory mechanisms of VCAM-1 and ICAM-1 induction and subsequent monocyte adhesion in TNF-treated human umbilical vein endothelial cells (HUVECs). PDTC or N-acetylcysteine dose dependently reduced TNF-induced VCAM-1 but not ICAM-1 surface protein (also in human umbilical arterial endothelial cells) and mRNA expression (by 70% at 100 μmol/L PDTC) in HUVECs as assessed by flow cytometry and polymerase chain reaction. Gel-shift analysis in HUVECs demonstrated that PDTC prevented NF-κB mobilization by TNF, suggesting that only VCAM-1 induction was controlled by NF-κB. Since HUVECs released superoxide anions in response to TNF, and H$_2$O$_2$ induces VCAM-1, PDTC may act as a radical scavenger. Although ICAM-1 induction was unaffected, inhibitors of NADPH oxidase (apocynin) or cytochrome P-450 (SKF525a) suppressed VCAM-1 induction by TNF, revealing that several radical-generating systems are involved in its regulation. PDTC, apocynin, or SKF525a decreased adhesion of monocytic U937 cells to TNF-treated HUVECs (by 75% at 100 μmol/L PDTC). Inhibition by anti-VCAM-1 monoclonal antibody 1G11 indicated that U937 adhesion was VCAM-1 dependent and suppression by antioxidants was due to reduced VCAM-1 induction. In conclusion, our data reflect a major contribution of NF-κB activation to the mediation of VCAM-1-dependent monocyte adhesion in stimulated HUVECs. Antioxidants may represent a new approach in the treatment of conditions related to increased VCAM-1 expression. (Arterioscler Thromb. 1994;14:1665-1673.)

Key Words • dithiocarbamate • human umbilical vein endothelial cells • nuclear factor-κB • tumor necrosis factor • vascular cell adhesion molecule-1

Tumor necrosis factor-α (TNF) potently stimulates leukocyte adhesion to human umbilical vein endothelial cells (HUVECs) by inducing various adhesion molecules, which exert distinct functions in leukocyte recruitment. Vascular cell adhesion molecule-1 (VCAM-1), an endothelial receptor for lymphocytes, may be involved in monocyte adhesion by interacting with the β$_i$-integrin very late activation antigen-4. TNF also increases expression of intercellular adhesion molecule-1 (ICAM-1), a ligand for β$_i$-integrins, and endothelial leukocyte adhesion molecule-1 (ELAM-1). Whereas ELAM-1 may account for polymorphonuclear leukocyte rolling and ICAM-1 mediates subsequent attachment, spreading, and migration, VCAM-1 may preferentially contribute to monocyte adhesion. Two TNF receptor types, R55 and R75, are present on HUVECs. TNF-induced adhesion is controlled by R55, correlating with a dominant role for R55 in TNF-dependent upregulation of VCAM-1, ICAM-1, and ELAM-1 and specific activation of nuclear factor-κB (NF-κB), a pleiotropic mediator of gene induction.

Structural and deletion analyses show that putative NF-κB binding sites in the promoters of VCAM-1, ICAM-1, and ELAM-1 genes are required for their induction by cytokines. NF-κB binds as a heterodimer of protein p50, processed from p105, and the transcriptional active subunit p65 to the 5' (p50) and 3' (p65) half site of a consensus palindromic recognition sequence GGGRNNYYCC. Both components are inducible by TNF and act in concert to optimally stimulate VCAM-1 gene transcription in HUVECs. NF-κB is retained in the cytoplasm as an inactive complex by 1κB protein. Various activators cause NF-κB release and nuclear translocation by alteration of 1κB, such as phosphorylation and oxidative or proteolytic degradation. TNF induces ICAM-1, ELAM-1, or VCAM-1 without activation, membrane translocation, or depletion of protein kinase C (PKC) in HUVECs, indicating that NF-κB mobilization by TNF can occur independently of PKC. Since HUVECs exhibit increased superoxide anion (O$_2^−$) production in response to cytokines, a recently suggested role for reactive oxygen intermediates (ROIs) as intracellular messen-
Cells (2 x 10^6) were treated for 30 minutes with saturating amounts of mouse anti-ICAM-1 monoclonal antibody (mAb) 84H10 (Dianova), anti–VCAM-1 mAb, anti–platelet endothelial cell adhesion molecule-1 (PECAM-1) mAb, anti–ELAM-1 mAb (endothelial cells), anti–CD11b mAb, and anti–CD49d mAb (U937) or IgG₃, isotype control (all from Camon) in PBS containing 0.5% bovine serum albumin (BSA) on ice. For staining, cells were reacted with goat anti-mouse fluorescein isothiocyanate IgG₃, (Camon). To avoid unspecific binding, cells were preincubated with 5% human serum in PBS for 15 minutes on ice. Samples were washed twice with fluorescence-activated cell sorter (Becton Dickinson), fixed in 2% paraformaldehyde to prevent homotypic aggregation, and analyzed with 10,000 cells per sample in a fluorescence-activated cell sorter (Becton Dickinson). After correction for unspecific binding (isotype control), specific mean fluorescence intensity (sMFI) was expressed in channels.

**Production of Superoxide Anions**

Secretion of O₂⁻ by cells was determined as superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c, as described. Detached HUVECs (10³/mL) were stimulated with TNF (100 U/mL) for 30 minutes at 37°C in PBS containing 80 mmol/L ferricytochrome c from horse heart (type V1) with or without 300 U/mL SOD from bovine erythrocytes. Cells were pelleted, and the absorbance of supernatants was determined at 550 nm. Production of O₂⁻ was calculated from the difference of absorbance between paired samples with or without SOD using an extinction coefficient of 21.1 mmol/L⁻¹·cm⁻¹ for reduced cytochrome c as nanomoles of O₂⁻ released by 10³ cells in 30 minutes.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared by ultrasonication disruption of cell membranes followed by high salt extraction with Dignman's buffer C and diluted with buffer D, as described. Nuclear protein (10 μg) was mixed with a double-stranded oligonucleotide corresponding to the NF-κB binding motif located −57 bp from the initiation site in the VCAM-1 promoter. Oligonucleotides were synthesized according to the published sequence (cs 5'-TGAAGGAGTTCC-3') and labeled with [³²P]dATP using Klenow DNA polymerase. A κB motif from the mouse IgH enhancer (5'-AGCTCCAGGGATTC-CCAGAGG-3') and an AP-1 motif from the collagenase enhancer (5'-TCAGGTCTGACTCATCTA-3') were used as competitor DNA. For binding for 15 minutes in the presence of 105 U/mL Tris (pH 7.5), 5% glycerol, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 50 mmol/L NaCl, 0.1% NP-40, 1 mg/mL BSA, 1 μg dl/dc, and 50,000 cpm [³²P]-labeled DNA, samples were separated on a nondenaturating 4% polyacrylamide gel, which was exposed to x-ray film overnight.

**Polymerase Chain Reaction**

Total RNA was isolated from 10³ cells per sample; lysed by NP-40; denatured in 7 mol/L urea, 1% sodium dodecyl sulfate, 10 mmol/L EDTA, and 10 mmol/L Tris-Cl buffer, then extracted with phenol/chloroform/isooamyl alcohol (50:50:1); and precipitated in 100% ethanol. cDNA was produced from each RNA extract by murine leukemia virus reverse transcriptase (GIBCO-BRL). Specific oligonucleotide primers were synthesized (Dr Arnold, Martinsried, FRG) and purified by high-performance liquid chromatography (HPLC) according to reported cDNA sequences [15,16] to yield polymerase chain reaction (PCR) products of the following lengths: 441 bp (VCAM-1), 413 bp (ICAM-1), and 548 bp (β-actin). Primers were selected from regions with minimal homology to exclude amplification of related sequences and to span introns for distinction from genomic DNA. cDNA was amplified by 32 cycles using Taq polymerase (Perkin-Elmer Cetus) in a Perkin-Elmer thermocycler 480 set to the following profile: 95°C melting (30 seconds), 58°C annealing (60 seconds), and 72°C extension (60 seconds). Amplification was linear with 32 cycles. PCR products (20 μL) applied to ethidium bromide–stained 1.5% agarose gels and separated by electrophoresis, were of predicted lengths, as determined by comigration of molecular weight markers. UV-illuminated gels were photographed with Polaroid 667 film and analyzed by reflection densitometry compared with quantification by HPLC (Abimed-Gilson). Peaks appeared at retention times corresponding to specific PCR products, as assessed by standards. Areas were determined by integration. Respective mRNA amounts were normalized to levels of β-actin mRNA, which served as internal standard to compensate for variations in mRNA extractions.

**U937 Cell Adhesion Assay**

HUVECs were seeded in 24-well plates 48 hours before the experiment. Only confluent HUVEC monolayers were

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**Methods**

**Cell Culture**

HUVECs and human umbilical arterial endothelial cells (HUAECs) were obtained from human umbilical cord veins or arteries, respectively, by digestion with chymotrypsin and were cultured in low-serum endothelial cell growth medium (Promo Cell) using T-75 flasks precoated with collagen in a 5% CO₂ atmosphere at 37°C, as described. HUVEC purity was assessed by morphology (cobblestone) and factor VIII staining. Confluent HUVECs (passage 2 or 3) were detached by 0.01% trypsin/EDTA and treated with TNF (8.7 x 10⁶ U/mL in phosphate-buffered saline [PBS] provided by BASF), interferon gamma (Abimed-Gilson). Peaks appeared at retention times corresponding to specific PCR products, as assessed by standards.

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Total RNA was isolated from 10³ cells per sample; lysed by NP-40; denatured in 7 mol/L urea, 1% sodium dodecyl sulfate, 10 mmol/L EDTA, and 10 mmol/L Tris-Cl buffer, extracted with phenol/chloroform/isooamyl alcohol (50:50:1); and precipitated in 100% ethanol. cDNA was produced from each RNA extract by murine leukemia virus reverse transcriptase (GIBCO-BRL). Specific oligonucleotide primers were synthesized (Dr Arnold, Martinsried, FRG) and purified by high-performance liquid chromatography (HPLC) according to reported cDNA sequences [15,16] to yield polymerase chain reaction (PCR) products of the following lengths: 441 bp (VCAM-1), 413 bp (ICAM-1), and 548 bp (β-actin). Primers were selected from regions with minimal homology to exclude amplification of related sequences and to span introns for distinction from genomic DNA. cDNA was amplified by 32 cycles using Taq polymerase (Perkin-Elmer Cetus) in a Perkin-Elmer thermocycler 480 set to the following profile: 95°C melting (30 seconds), 58°C annealing (60 seconds), and 72°C extension (60 seconds). Amplification was linear with 32 cycles. PCR products (20 μL) applied to ethidium bromide–stained 1.5% agarose gels and separated by electrophoresis, were of predicted lengths, as determined by comigration of molecular weight markers. UV-illuminated gels were photographed with Polaroid 667 film and analyzed by reflection densitometry compared with quantification by HPLC (Abimed-Gilson). Peaks appeared at retention times corresponding to specific PCR products, as assessed by standards. Areas were determined by integration. Respective mRNA amounts were normalized to levels of β-actin mRNA, which served as internal standard to compensate for variations in mRNA extractions.
used, as confirmed by microscopic inspection. Fresh medium was provided before stimulation with TNF (100 U/mL). U937 cells (3 x 10^6) were incubated in RPMI 1640 medium (6 mL) containing 2% FCS and 10 μg/mL of the fluorescence dye 2'7' bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Boehringer Mannheim) at 37°C for 30 minutes. Dye loading was stopped by addition of 44 mL RPMI 1640 with 2% FCS. Fluorescence-labeled cells were resuspended (10^6/mL) in medium 199 with 10 mmol/L HEPES buffer (M199H, GIBCO-BRL). HUVECs were washed with M199H before addition of loaded U937 cells and incubated at 37°C, 5% CO₂, and 90% humidity. After 30 minutes, the U937 suspension was withdrawn, HUVECs were gently washed twice with M199H, and inverted plates were centrifuged (50g, 4°C, 5 minutes). Cells were lysed with 0.1% Triton X-100 in 0.1 mol/L Tris buffer per well (pH 8), and fluorescence was measured by a PTI Deltascan spectrofluorometer (excitation, 485 nm; emission, 535 nm). Adherent cells per well were calculated by comparing the determined fluorescence to a standard curve of BCECF activity per cell, expressed as percent adhesion of added U937 cells per well. Some HUVECs were treated with saturating amounts of anti-VCAM-1 mAb 1G11 (Dianova), which has been shown to block lymphocyte adhesion to HUVECs, 30 minutes before addition and during adhesion of U937 cells.

All other reagents were from Sigma Chemical Co.

Statistics
Data were analyzed by Student’s t test.

Results
Characterization of Cell Adhesion Molecule (CAM) Induction by TNF
We studied expression of VCAM-1 and ICAM-1 by TNF-treated HUVECs using flow cytometry. Although VCAM-1 was not constitutively expressed by HUVECs, it was markedly induced after stimulation with TNF (100 U/mL) for 24 hours (Fig 1) to an sMFI of 154±13 channels. In contrast, ICAM-1 was already expressed (sMFI, 49±10 channels) by untreated HUVECs and dramatically upregulated by TNF (100 U/mL, 24 hours). An sMFI of 398±21 channels reflected a 20-fold increase in antigen density (Fig 1). The induction of CAM by TNF was dose dependent and maximal at 100 U/mL. Cytotoxicity was ruled out by determination of constitutive PECAM-1 expression, which was clearly diminished in avital cells treated with toxic concentrations of H₂O₂ but not affected by TNF or thiol compounds (Table 1). Consistent with previous observations, ELAM-1 induction by TNF was only temporary and almost undetectable after treatment with TNF for 24 hours (not shown). Induction of VCAM-1 and ICAM-1 by TNF exhibited different kinetics (Fig 2). Suppression of enhanced CAM expression by the protein synthesis inhibitor cycloheximide (10 μg/mL) and actinomycin D (10 μg/mL) indicates that induction of both VCAM-1 and ICAM-1 by TNF depends on de novo protein synthesis and transcription (not shown). HUVEC stimulation with interferon gamma (100 U/mL) for 24 hours resulted in lower enhancement of ICAM-1 expression compared with TNF and did not induce VCAM-1 (Fig 1).

Effect of the Thiol Compounds PDTC and NAC on VCAM-1 Induction by TNF
Since NF-κB binding motifs have been identified in VCAM-1 and ICAM-1 promoters, 10,11 we used PDTC, a selective inhibitor of NF-κB activation, to analyze the signal transduction pathways mediating CAM induction by TNF. PDTC was shown to block mobilization of NF-κB in TNF-stimulated T cells 22 and lipopolysaccharide-stimulated monocytes, whereas binding of the nuclear factors oct-1, CREB, SP-1, and C/EBP was not affected. Pretreatment of HUVECs with PDTC for 24 hours before stimulation with TNF (100 U/mL) for 24 hours resulted in biphasic, dose-dependent prevention of VCAM-1 induction by TNF. Inhibition was first evident at 10 μmol/L PDTC, maximal at 100 μmol/L PDTC, and partially relieved at 300 μmol/L PDTC (Table 2). A reduction of sMFI to 38±15 channels by 100 μmol/L PDTC reflected a 66% decrease in the surface density of VCAM-1 protein. The effect was rapid in onset, since simultaneous addition of PDTC with TNF was equally effective, whereas addition of PDTC (100 μmol/L) 3 hours after TNF suppressed VCAM-1 induction to an sMFI of 65±5 channels. Although 100 μmol/L pyrrolidine had no effect, the derivative diethyldithiocarbamate was also effective (not shown). Pretreatment with NAC, which blocks NF-κB activation by TNF, 22 for 24 hours also dose-dependently suppressed VCAM-1 induction by TNF.

Fig 1. Plots show flow cytometry in human umbilical vein endothelial cells stimulated by tumor necrosis factor-α (TNF) or interferon (IFN). Cells were stimulated with TNF or IFN at indicated concentrations for 24 hours; stained for vascular cell adhesion molecule-1 (VCAM-1) (left), intercellular adhesion molecule-1 (ICAM-1) (right), or isotype control (dotted lines, reflecting unspecific binding); and analyzed by flow cytometry. Shown are original histograms representative of four similar experiments. Relative cell numbers (vertical) and fluorescence intensity on a log₁₀ scale (horizontal) are indicated.
TABLE 1. Expression of Platelet Endothelial Cell Adhesion Molecule-1 in Human Umbilical Vein Endothelial Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PECAM-1 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>222±34</td>
</tr>
<tr>
<td>TNF</td>
<td>218±38</td>
</tr>
<tr>
<td>PDTC</td>
<td>223±27</td>
</tr>
<tr>
<td>NAC</td>
<td>223±36</td>
</tr>
<tr>
<td>TNF+PDTC</td>
<td>226±44</td>
</tr>
<tr>
<td>TNF+NAC</td>
<td>225±39</td>
</tr>
<tr>
<td>H2O2</td>
<td>122±10</td>
</tr>
</tbody>
</table>

PECAM-1 indicates platelet endothelial cell adhesion molecule-1; TNF, tumor necrosis factor-α; PDTC, pyrrolidine dithiocarbamate; and NAC, N-acetylcysteine. Cells were pretreated with or without PDTC (100 μmol/L) or NAC (10 mmol/L) for 24 hours and stimulated with TNF (100 U/mL) or H2O2 (1 mmol/L) for 12 hours. Cells were stained for PECAM-1 or isotype control and analyzed by flow cytometry. Data are expressed as specific mean fluorescence intensity corrected for autofluorescence/unspecific binding in channels and represent mean±SD of four separate experiments performed in duplicate.

The effect was first significant at 1 mmol/L and maximal at 10 mmol/L (Table 2). Differences in ID50 for NAC and PDTC may be due to less effective cellular absorption of NAC. In contrast, TNF-stimulated ICAM-1 expression was slightly attenuated by PDTC only at 100 μmol/L (sMFI, 362±15 channels), indicating that NF-κB activation is essential for VCAM-1 but not ICAM-1 induction by TNF. Pretreatment of HUAECs with 100 μmol/L PDTC for 24 hours also resulted in marked inhibition of VCAM-1 induction by TNF, whereas ICAM-1 induction was unaffected (Fig 3). This suggests that HUVECs as well as HUAECs may represent appropriate model systems for studying regulatory mechanisms of CAM induction.

![Figure 2](image)

**Fig 2.** Line graph shows kinetics of vascular cell adhesion molecule-1 (VCAM-1) induction in human umbilical vein endothelial cells by tumor necrosis factor-α (TNF). Cells were stimulated with 100 U/mL TNF for 1, 3, 6, 12, 24, or 48 hours; stained for VCAM-1, intercellular adhesion molecule-1 (ICAM-1), or isotype control; and analyzed by flow cytometry. Data are expressed as specific mean fluorescence intensity (sMFI) corrected for unspecific binding in channels. Shown is one representative of four similar experiments.

![Figure 3](image)

**Fig 3.** Plots show effect of pyrrolidine dithiocarbamate (PDTC) on vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) induction by tumor necrosis factor-α (TNF) in human umbilical arterial endothelial cells. Cells were pretreated with or without PDTC (100 μmol/L) for 24 hours and stimulated with TNF (100 U/mL) for 24 hours; stained for VCAM-1, ICAM-1, or isotype control (reflecting unspecific binding, NC); and analyzed by flow cytometry. Shown are original histograms representative of three similar experiments. Relative cell numbers (vertical) and fluorescence intensity on a log10 scale (horizontal) are indicated.

TABLE 2. Effect of PDTC and NAC on VCAM-1 Induction by TNF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>VCAM-1 Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF + PDTC</td>
<td>0 μmol/L</td>
<td>154±13</td>
</tr>
<tr>
<td></td>
<td>10 μmol/L</td>
<td>125±20*</td>
</tr>
<tr>
<td></td>
<td>30 μmol/L</td>
<td>89±16f</td>
</tr>
<tr>
<td></td>
<td>100 μmol/L</td>
<td>38±15f</td>
</tr>
<tr>
<td></td>
<td>300 μmol/L</td>
<td>66±12f</td>
</tr>
<tr>
<td>TNF + NAC</td>
<td>0 mmol/L</td>
<td>154±13</td>
</tr>
<tr>
<td></td>
<td>1 mmol/L</td>
<td>130±14*</td>
</tr>
<tr>
<td></td>
<td>3 mmol/L</td>
<td>97±25f</td>
</tr>
<tr>
<td></td>
<td>10 mmol/L</td>
<td>35±10f</td>
</tr>
</tbody>
</table>

PDTC indicates pyrrolidine dithiocarbamate; NAC, N-acetylcysteine; VCAM-1, vascular cell adhesion molecule-1; and TNF, tumor necrosis factor-α. Human umbilical vein endothelial cells were pretreated with or without PDTC or NAC at indicated concentrations for 24 hours and stimulated without (control) or with TNF (100 U/mL) for 12 hours. Cells were stained for VCAM-1 or isotype control and analyzed by flow cytometry. Data are expressed as specific mean fluorescence intensity (sMFI) corrected for unspecific binding in channels and represent mean±SD of four separate experiments performed in duplicate.

Induction of VCAM-1 mRNA Expression Determined by PCR

In unstimulated HUVECs, VCAM-1 mRNA was hardly detectable, but stimulation with TNF (100
Fig 4. Photograph of UV-illuminated gel shows effect of pyrrolidine dithiocarbamate (PDTC) on tumor necrosis factor-α (TNF)-induced cell adhesion molecule mRNA expression in human umbilical vein endothelial cells. Cells were pretreated with or without PDTC (100 μmol/L) for 24 hours and stimulated with or without TNF (100 U/mL) for 12 hours. Total RNA was extracted, and equal amounts were reverse transcribed. cDNA was amplified by polymerase chain reaction using specific primers for β-actin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1); 20 μL of polymerase chain reaction products or molecular weight standards (M) was applied to ethidium bromide-stained 1.5% agarose gels and separated by electrophoresis. Shown is a gel photograph representative of three similar experiments.

TABLE 3. Expression of VCAM-1 and ICAM-1 mRNA with PDTC for 24 hours (Fig 4). Both HPLC analysis of PCR products and laser densitometry of VCAM-1 bands revealed a significant 70% suppression of TNF-specific VCAM-1 PCR products. This induction of VCAM-1 indicates vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; TNF, tumor necrosis factor-α; and PDTC, pyrrolidine dithiocarbamate. Human umbilical vein endothelial cells were pretreated with or without PDTC (100 μmol/L) for 24 hours and stimulated with or without TNF (100 U/mL) for 12 hours. mRNA was extracted, reverse transcribed, and amplified by polymerase chain reaction (PCR) using specific primers for β-actin, VCAM-1, and ICAM-1. PCR products were quantified by high-performance liquid chromatography and normalized to β-actin mRNA expression as internal standard. Data indicate specific PCR products expressed as percent in relation to β-actin mRNA (100%) and represent mean±SD of three separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VCAM-1</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>29±15</td>
</tr>
<tr>
<td>PDTC</td>
<td>0</td>
<td>46±24</td>
</tr>
<tr>
<td>TNF</td>
<td>57±16*</td>
<td>162±27*</td>
</tr>
<tr>
<td>TNF+PDTC</td>
<td>15±8†</td>
<td>119±30</td>
</tr>
</tbody>
</table>

VCAM-1 indicates vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; TNF, tumor necrosis factor-α; and PDTC, pyrrolidine dithiocarbamate. Human umbilical vein endothelial cells were pretreated with or without PDTC (100 μmol/L) for 24 hours and stimulated with or without TNF (100 U/mL) for 12 hours. mRNA was extracted, reverse transcribed, and amplified by polymerase chain reaction (PCR) using specific primers for β-actin, VCAM-1, and ICAM-1. PCR products were quantified by high-performance liquid chromatography and normalized to β-actin mRNA expression as internal standard. Data indicate specific PCR products expressed as percent in relation to β-actin mRNA (100%) and represent mean±SD of three separate experiments.

*P<.01 vs control.
†P<.01 vs TNF.

The reported antioxidant-responsive induction of the transcription factor AP-1, which binds to a motif present in the ICAM-1 promoter. Neither TNF nor PDTC altered constitutive β-actin transcription.

Subsequently, we evaluated whether PDTC inhibited VCAM-1 gene transcription by blocking activation of NF-κB-like regulatory factors. Gel-shift analysis demonstrated a marked induction of NF-κB-like DNA binding activity in response to stimulation with TNF for 1 hour and almost complete prevention of TNF-stimulated NF-κB mobilization by pretreatment of HUVECs with 100 μmol/L PDTC for 24 hours (Fig 5). SKF525a (5 μmol/L), an inhibitor of cytochrome P-450, was less effective (Fig 5). Competition studies with a prototypic NF-κB motif revealed specific binding, whereas an irrelevant motif had no effect (not shown). These data suggest that PDTC suppresses VCAM-1 induction by blocking NF-κB activation and VCAM-1 transcription.

Role of ROIs in VCAM-1 Induction in HUVECs

NF-κB activation can occur independently of PKC. In our system, staurosporine (1 nmol/L), a potent inhibitor of PKC, did not suppress TNF-induced VCAM-1 upregulation (not shown). This concentration did not affect protein synthesis. PDTC and NAC exert antioxidative activity by scavenging radicals, raising reduced glutathione levels, and providing reduced glutathione peroxidase with cosubstrate required for elimination of ROIs. To confirm the relevance of these effects and to investigate whether ROIs are involved in mediating VCAM-1 induction via NF-κB, as indicated by inhibitory effects of PDTC, we studied O2⁻ secretion.
in HUVECs. Stimulation with TNF (100 U/mL) resulted in a significant increase of basal $O_2^-$ release ($P<.01$, $n=3$) from 2.0±0.3 to 7.2±1.1 nmol/10^6 cells per 30 minutes, suggesting a possible role for $O_2^-$ in NF-$\kappa$B-mediated VCAM-1 induction by TNF. SKF525a and Apocynin on VCAM-1 Induction by TNF (Fig 6). This cell line expresses very late activation antigen-4 (86±7% positive; sMFI, 133±13 channels; $n=4$), which serves as a VCAM-1 ligand, but not CD11b, the receptor for ICAM-1. Stimulation of HUVECs with TNF.

### Table 4. VCAM-1 Induction by $H_2O_2$ and Effect of SKF525a and Apocynin on VCAM-1 Induction by TNF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VCAM-1 Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6±5</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>26±3*</td>
</tr>
<tr>
<td>TNF</td>
<td>154±13</td>
</tr>
<tr>
<td>TNF+APO</td>
<td>99±11†</td>
</tr>
<tr>
<td>TNF+SKF</td>
<td>108±9†</td>
</tr>
<tr>
<td>TNF+AL</td>
<td>156±10</td>
</tr>
<tr>
<td>TNF+APO+SKF</td>
<td>62±28†</td>
</tr>
</tbody>
</table>

VCAM-1 indicates vascular cell adhesion molecule-1; TNF, tumor necrosis factor-$\alpha$; APO, apocynin; SKF, SKF525a; and AL, allopurinol. Human umbilical vein endothelial cells were treated with or without specific inhibitors of NADPH oxidase (100 $\mu$g/mL APO), cytochrome P-450 (5 $\mu$mol/L SKF), or xanthine oxidase (30 $\mu$mol/L AL) for 24 hours and stimulated with TNF (100 U/mL) for 12 hours or $H_2O_2$ (50 $\mu$mol/L) for 6 hours. Cells were stained for VCAM-1 or isotype control and analyzed by flow cytometry. Data are expressed as specific mean fluorescence intensity corrected for unspecific binding in channels and represent mean±SD of four separate experiments performed in duplicate.

* $P<.01$ vs control.
† $P<.01$ vs TNF.

... indicate that NF-$\kappa$B-mediated VCAM-1 expression follows activation by multiple radical-generating systems. Interestingly, the lipophilic antioxidant vitamin E had no comparable effect, excluding unspecific antioxidative actions of SKF525a or apocynin in the plasma membrane. None of the antioxidants altered basal or TNF-stimulated ICAM-1 expression.

### Inhibition of U937 Cell Adhesion to TNF-Treated HUVECs by PDTC

To study the functional consequences of our results, we analyzed VCAM-1-dependent adhesion to TNF-treated HUVECs. As an appropriate model to show the relevance of modulated VCAM-1 induction, we used human premonocytic U937 cells for the adhesion assay. This cell line expresses very late activation antigen-4 (86±7% positive; sMFI, 133±13 channels; $n=4$), which serves as a VCAM-1 ligand, but not CD11b, the receptor for ICAM-1. Stimulation of HUVECs with TNF...
TNF (100 U/mL) for 24 hours enhanced U937 adhesion (Fig 7). Conversely, erythroleukemic K562 cells, which totally lack monocyte-specific very late activation antigen-4 expression, did not exhibit enhanced adhesion to TNF-stimulated HUVECs (not shown). A stimulation period of 24 hours was selected to exclude involvement of ELAM-1, which was practically absent after 24 hours. The major contribution of VCAM-1 to U937 cell adhesion is also evident from our finding that basal adhesion to unstimulated HUVECs was negligible despite constitutive ICAM-1 expression. Moreover, stimulation of HUVECs with interferon gamma (100 U/mL) induced ICAM-1 but not VCAM-1 expression and only marginally affected U937 cell adhesion (3.5% versus 2.6%). This suggests that not interaction of CD11b with ICAM-1 but interaction of very late activation antigen-4 with VCAM-1 was important in our assay. Adhesion to stimulated HUVECs increased with TNF concentration (Fig 7), parallel to TNF-induced VCAM-1 expression, as shown by flow cytometry (Fig 1). VCAM-1 dependence of U937 cell adhesion was further confirmed by preincubation with blocking anti-VCAM-1 mAb 1G11 29 (Fig 7). Incomplete blockade (40%) may result from mAb properties or partial involvement of other yet unidentified adhesion molecules. Although PDTC alone had no effect (2.5%), pretreatment of HUVECs with 100 μmol/L PDTC for 24 hours caused marked inhibition of TNF-induced adhesion (75%). SKF525a (5 μmol/L) or apocynin (100 μg/mL) was also effective (Fig 7), consistent with their suppressive effect on VCAM-1 induction (Fig 6). PDTC exhibited a similar biphasic dose dependence, as observed for prevention of NF-κB mobilization 32 and VCAM-1 induction (Fig 2). Thus, our data demonstrate that reduction of TNF-stimulated adhesion by PDTC or SKF525a is probably caused by blockade of NF-κB mobilization and subsequent VCAM-1 transcription.

Discussion

κB-like elements are present in regulatory sequences of VCAM-1 and ICAM-1 genes. 10,11 To analyze the role of NF-κB in mediating CAM induction by TNF in HUVECs and HUAECs, we used the radical-scavenging thiol compound PDTC to test its potential inhibitory action on VCAM-1 and ICAM-1 protein synthesis and mRNA expression and function. In monocytes and T cells, PDTC selectively inhibited NF-κB activation but did not affect binding of other nuclear factors, such as SP-1, oct-1 or CREB, in response to TNF and other stimuli. 22,30 In HeLa cells, PDTC rather induced AP-1 transactivation and DNA binding. 31 In HUVECs, PDTC almost completely blocked TNF-stimulated NF-κB mobilization. Although VCAM-1 induction was potently prevented by PDTC at both the surface protein and mRNA levels, ICAM-1 induction was only slightly affected, suggesting that activation of NF-κB–like regulatory factors is responsible for VCAM-1 but not ICAM-1 induction by TNF in HUVECs. Consistent with these results, both κB sites in the VCAM-1 enhancer are required for optimal gene expression. 14 The suppressive effect of PDTC on VCAM-1 induction by TNF reveals a biphasic dose-response curve with relieved inhibition at 300 μmol/L, which exactly resembles the biphasic dose-response relation observed for suppression of NF-κB mobilization by PDTC in T cells. A biphasic dose dependence may also reflect oxidative properties of thiols at concentrations greater than 100 μmol/L. 36 Based on this characteristic dose response, interference of PDTC with TNF receptor or DNA binding activity of NF-κB and disintegration of cells appear unlikely, indicating a specific action of PDTC on NF-κB–mediated VCAM-1 induction. At high concentrations, p55 alone stimulates the VCAM-1 promoter in HUVECs; at low concentrations it acts in concert with p50. 15 Selectivity may be determined by dimeric association of specific NF-κB subunits, allowing differential regulation of genes containing κB sites. 37 Elements flanking κB sites may interact with activated NF-κB to modulate initiation of transcription from distinct promoters. This may explain why NF-κB appears to be essential for VCAM-1 but not sufficient for ICAM-1 and ELAM-1 induction. 12 In addition, different kinetics of induction may reflect distinct mechanisms for the regulation of VCAM-1 and ICAM-1 expression. This may be due to posttranscriptional stabilization of ICAM-1 mRNA. 38

Cultured arterial endothelial cells have been reported to express VCAM-1 and increased ICAM-1 after treatment with lysophosphatidylcholine, a component of atherogenic lipoproteins. 39 Since TNF induced a similar pattern of VCAM-1 and ICAM-1 expression in HUAECs, the preventive effect of PDTC on VCAM-1 induction by TNF in arterial endothelial cells demonstrates that the results obtained in HUVECs may be relevant to study the induction and function of CAMs involved in atherogenesis.

In our system, VCAM-1 induction by TNF occurred independently of PKC activation. Together with reported data that neither TNF nor PDTC affects PKC, 17,21 this implies that PDTC rather acts by interfering with radicals. To further analyze the molecular mechanisms of NF-κB–mediated VCAM-1 induction by TNF, we focused on a recently suggested role of ROS in the mediation of NF-κB activation. 21,40 Importantly, we demonstrated that HUVECs produced O2•− in response to TNF, indicating a possible role of O2•− in mediating NF-κB mobilization and subsequent VCAM-1 induction. PDTC has been described to be more potent than NAC in T cells and has additional metal-chelating properties. Since metal ions participate in the conversion of H2O2 to OH• by the Fenton reaction, OH• was proposed to be mainly responsible for NF-κB activation. However, H2O2 only slightly enhanced VCAM-1 expression in HUVECs, and NAC was equally effective in preventing VCAM-1 induction by TNF in our experiments. This indicates that PDTC exerts its effects predominantly by scavenging radicals and altering intracellular thiol levels. 32 Consistent with our results, the PDTC-induced inhibition of NF-κB mobilization in monocytes was not reversible by Co2+ or Mn2+ cations, which block the suppression of NF-κB mobilization by the metal chelator orthophenanthroline. 30

The slight enhancement of HUVEC VCAM-1 expression by H2O2 suggests that besides O2•−, other mediators have to contribute to maximal VCAM-1 induction. In monocytes, elements of NADPH oxidase appear not to be required for NF-κB activation. 40 Since HUVECs can generate radicals by various enzymes, such as NADPH oxidase, 20 xanthine oxidase, 33 or cytochrome P-450-dependent oxidase, 24 we assessed their conceivable involvement in VCAM-1 induction by TNF. In neutro-
philms stimulated to release ROIs and peroxidase, the plant phenol apocynin is activated to metabolites that prevent assembly of functional, $O_2^*$-generating NADPH oxidase in the membrane by conjugation to thiol groups.\textsuperscript{39} Inhibition by apocynin has implicated NADPH oxidase in lipopolysaccharide-induced ELAM-1 mRNA expression in HUVECs.\textsuperscript{41} However, components of phagocyte-type NADPH oxidase remain to be demonstrated in HUVECs. In our experiments, apocynin only partly inhibited VCAM-1 induction by TNF, providing further evidence for additional mechanisms required for complete induction.

In stimulated HUVECs, low-density lipoprotein increased cytochrome P-450-dependent synthesis of 14,15-epoxyeicosatrienoic acid (14,15-EET),\textsuperscript{24} which promotes U937 adhesion to HUVECs.\textsuperscript{2} Moreover, oxidized low-density lipoprotein may influence gene expression by causing oxidative stress and NF-$\kappa$B activation.\textsuperscript{43} SKF525a, which prevented 14,15-EET synthesis in HUVECs,\textsuperscript{24} clearly reduced NF-$\kappa$B mobilization and VCAM-1 induction by TNF in our system. Thus, 14,15-EET may induce VCAM-1 expression in HUVECs after NF-$\kappa$B mobilization and consequently explain enhanced U937 cell adhesion.\textsuperscript{42} Since NF-$\kappa$B activation by TNF was found to be controlled by ROIs derived from the respiratory chain in fibrosarcoma cells, as demonstrated by depletion of mitochondrial electron transport, SKF525a may act by interference with electron entry to mitochondrial ubiquinone pools.\textsuperscript{44} In T cells, esterified vitamin E derivatives, but not vitamin E, inhibit TNF-induced NF-$\kappa$B mobilization, implying that oxidative membrane processes required for cell signaling are localized in compartments accessible only to vitamin E derivatives.\textsuperscript{45} Taken together, the data suggest that several intracellular radical-generating systems are involved in mediating NF-$\kappa$B activation and VCAM-1 induction by TNF. Whereas unspecific oxidation eliminates NF-$\kappa$B binding,\textsuperscript{46} ROIs have been proposed to selectively activate phosphorylation\textsuperscript{47} via a redox-regulated tyrosine kinase\textsuperscript{48} or to induce IkB degradation by a cytoplasmic protease.\textsuperscript{10} In accordance with our results with PDTC, neither $H_2O_2$ nor inhibitors of oxidative metabolism affected ICAM-1 expression, further indicating distinct mechanisms for VCAM-1 versus ICAM-1 induction.

To confirm the functional significance of inhibition of VCAM-1 protein and mRNA induction by PDTC, we established an adhesion assay using monocytic U937 cells, which express the VCAM-1 counter receptor very late activation antigen-4 but not the ICAM-1 ligand MAC-1 (CD11b/CD18). ICAM-1, which may account for polymorphonuclear neutrophil adhesion and migration,\textsuperscript{7} plays a minor role in our assay. So far, a partial involvement of VCAM-1 in peripheral blood mononuclear cell adhesion to HUVECs has been shown.\textsuperscript{2} Our model revealed a major contribution of VCAM-1 to the adhesion process in which U937 cells to TNF-stimulated HUVECs by inhibition experiments with mAb 1G11,\textsuperscript{29} emphasizing the importance of VCAM-1 for monocyte adhesion.\textsuperscript{6} Consequently, pretreatment with PDTC or SKF525a, which caused suppression of NF-$\kappa$B-mediated VCAM-1 induction, inhibited U937 monocyte adhesion to TNF-stimulated HUVECs.

Monocyte recruitment into the vascular wall follows adhesion to endothelial cells in response to a variety of noxious influences and is a crucial step in the pathogenesis of atherosclerosis.\textsuperscript{49} Immunohistochemical staining revealed VCAM-1 expression in aortic endothelium covering foam cell lesions or extending beyond their edges in hyperlipidemic mice,\textsuperscript{50} in areas of neovascularization and inflammatory infiltration of human coronary atherosclerotic plaques,\textsuperscript{33} after aortic balloon injury in rabbit vascular cells,\textsuperscript{52} and in allograft biopsies.\textsuperscript{53} Oxidative stress and subsequent NF-$\kappa$B activation have been implicated in the induction of endothelial genes associated with the initiation of atherosclerosis.\textsuperscript{44} Thus, prevention of NF-$\kappa$B-mediated VCAM-1 induction and monocyte adhesion by PDTC confirms an important contribution of radicals and NF-$\kappa$B mobilization to atherogenesis. In conclusion, our study provides a possibility of selectively modulating VCAM-1 induction and suggests additional therapeutic implications of antioxidants in inflammatory conditions related to enhanced VCAM-1 expression.

During the preparation of this manuscript, a study was published that is in accordance with our data (Marui et al\textsuperscript{50}).

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