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 H2O2 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 monocytes 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
gators mediating NF-κB activation\(^{21}\) may also contribute to TNF-induced VCAM-1 expression and subsequent monocyte adhesion in HUVECs.

To study the role of NF-κB in mediating VCAM-1 induction and monocyte adhesion to TNF-treated HUVECs, we used the metal chelator and radical-scavenging antioxidant pyrroldione dithiocarbamate (PDTC). PDTC potently and specifically inhibits NF-κB activation in response to various stimuli by suppressing the release of IkB in intact cells.\(^{22}\) We demonstrate that PDTC dose dependently prevents VCAM-1 mRNA expression and protein synthesis in TNF-stimulated HUVECs but only slightly affects ICAM-1 induction. Consequently, PDTC, inhibitors of NADPH oxidase (the plant phenol apocynin), or cytochrome P-450 (SKF525a) reduced VCAM-1-dependent adhesion of monotypic U937 cells to TNF-treated HUVECs.

**Methods**

**Cell Culture**

HUVECs and human umbilical arterial endothelial cells (HUAEs) 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% CO\(_2\) atmosphere at 37°C, as described.\(^{23}\) 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 grown in T-25 flasks for treatment with human recombinant TNF (8.7x10\(^4\) U/mL in phosphate-buffered saline [PBS] provided by BASF), interferon gamma (Peprotech), and hydrogen peroxide (H\(_2\)O\(_2\)); and allopurinol (in dimethyl sulfoxide), metapyrone (SKF525a, SmithKline Beecham), or 4-hydroxy-mercaptoethanol (apocynin, C. Roth, Karlsruhe, FRG; all dissolved in H\(_2\)O\(_2\)); and allopurinol (in dimethyl sulfoxide), cycloheximide, or actinomycin D at indicated concentrations and periods. For flow cytometry, RNA extractions, and O\(_2^-\) measurement, cells were harvested by careful treatment with 0.01% trypsin/EDTA and grown in 25-T flasks for treatment with human recombinant TNF (8.7x10\(^4\) U/mL in phosphate-buffered saline [PBS] provided by BASF), interferon gamma (Peprotech), and hydrogen peroxide (H\(_2\)O\(_2\)); and allopurinol (in dimethyl sulfoxide), cycloheximide, or actinomycin D at indicated concentrations and periods.

**Immunofluorescence**

Cells (2x10\(^5\)) 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 IgG\(_\text{1}\), isotype control (all from Amgen) in PBS containing 0.5% bovine serum albumin (BSA) on ice. For staining, cells were reacted with goat anti-mouse fluorescein isothiocyanate IgG\(_\text{1}\) (Camon). To avoid unspecified binding, cells were preincubated with 5% human serum in PBS for 15 minutes on ice. Samples were washed twice with fluorescence-activated cell sorter buffer (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 unspecified binding (isotype control), specific mean fluorescence intensity (mSFI) was expressed in channels.

**Production of Superoxide Anions**

Secretion of O\(_2^-\) by cells was determined as superoxide dismutase (SOD)–inhibitable reduction of ferriytochrome c, as described.\(^{25}\) Detached HUVECs (10\(^3\)/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 VI) 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 O\(_2^-\) 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 O\(_2^-\) released by 10\(^4\) cells in 30 minutes.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared by ultrasonic disruption of cell membranes followed by high salt extraction with Dignam's buffer C and diluted with buffer D, as described.\(^{26}\) 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.\(^{10}\) Oligonucleotides were synthesized according to the published sequence (cs 5'-TGAAGGGATTTCCC-3') and labeled with [\(^{32}\)P]dATP using Klenow DNA polymerase. A κB motif from the mouse IgH enhancer (5'-AGCCTGAGGGACCTC-GGAGG-3') and an AP-1 motif from the collagenase enhancer (5'-TCGAGGTGTCACTATGCTA-3') were used as competitor DNA. For binding for 15 minutes in the presence of 105). Trit (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/dtC, and 50,000 cpm [\(^{32}\)P]-labeled DNA, samples were separated on a nondenaturing 4% polyacrylamide gel, which was exposed to x-ray film overnight.

**Polymerase Chain Reaction**

Total RNA was isolated from 10\(^4\) cells per sample; lysed by NP-40; denaturated 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 1 μg of 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\(^{11,27}\) 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 (30 seconds). Amplification was linear with 32 cycles. PCR products (20 μL) applied to ethidium bromide–stained 1% agarose gels in 0.5% 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 densitometry.

**U937 Cell Adhesion Assay**

HUVECs were seeded in 24-well plates 48 hours before the experiment. Only confluent HUVEC monolayers were seeded in 24-well plates 48 hours before the experiment. Only confluent HUVEC monolayers were
used, as confirmed by microscopic inspection. Fresh medium was provided before stimulation with TNF (100 U/mL). U937 cells \((3 \times 10^5)\) were incubated in RPMI 1640 medium (6 mL) containing 2% FCS and 10 \(\mu\)g/mL of the fluorescence dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy-methyl 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 \((10^7/\mu\)L) 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% CO\(_2\), and 90% humidity. After 30 minutes, the U937 suspension was withdrawn, HUVECs were gently washed twice with M199H, and inverted plates were centrifuged (5k, 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,\(^6\) 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 H$_2$O$_2$ but not affected by TNF or thiol compounds (Table 1). Consistent with previous observations,\(^5\) 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 \(\mu\)g/mL) and actinomycin D (10 \(\mu\)g/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-\(\kappa\)B binding motifs have been identified in VCAM-1 and ICAM-1 promoters,\(^10,11\) we used PDTC, a selective inhibitor of NF-\(\kappa\)B activation, to analyze the signal transduction pathways mediating CAM induction by TNF. PDTC was shown to block mobilization of NF-\(\kappa\)B in TNF-stimulated T cells\(^22\) and lipopolysaccharide-stimulated monocytes,\(^30\) 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 \(\mu\)mol/L PDTC, maximal at 100 \(\mu\)mol/L PDTC, and partially relieved at 300 \(\mu\)mol/L PDTC (Table 2). A reduction of sMFI to 38±15 channels by 100 \(\mu\)mol/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 \(\mu\)mol/L) 3 hours after TNF suppressed VCAM-1 induction to an sMFI of 65±5 channels. Although 100 \(\mu\)mol/L pyrrolidine had no effect, the derivative diethyldithiocarbamate was also effective (not shown). Pretreatment with NAC, which blocks NF-\(\kappa\)B activation by TNF,\(^23\) for 24 hours also dose-dependently suppressed VCAM-1 induction by TNF.
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>H$_2$O$_2$</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 H$_2$O$_2$ (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 ID$_{50}$ 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.

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±16*</td>
</tr>
<tr>
<td></td>
<td>100 μmol/L</td>
<td>38±15*</td>
</tr>
<tr>
<td></td>
<td>300 μmol/L</td>
<td>66±12*</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±25*</td>
</tr>
<tr>
<td></td>
<td>10 mmol/L</td>
<td>35±10*</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...
TABLE 3. Expression of VCAM-1 and ICAM-1 mRNA with PDTC for 24 hours (Fig 4). Both HPLC analysis of 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>
<thead>
<tr>
<th>Treatment</th>
<th>VCAM-1</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>46±24</td>
</tr>
<tr>
<td>PDTC</td>
<td>48±15</td>
<td>162±27*</td>
</tr>
<tr>
<td>TNF</td>
<td>15±8†</td>
<td>199±30</td>
</tr>
<tr>
<td>TNF+PDTC</td>
<td>15±8†</td>
<td>199±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. Total RNA was extracted, and equal amounts were reverse transcribed. cDNA was amplified by polymerase chain reaction using specific primers for β-actin, VCAM-1, and ICAM-1. PCR products were quantified by PCR products and laser densitometry of VCAM-1 PCR products.

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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. PTDC 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 O$_2^-$ secretion.
TABLE 4. VCAM-1 Induction by H$_2$O$_2$ 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$_2$O$_2$</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>106±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-α; APO, apocynin; SKF, SKF525a; and AL, allopurinol. Human umbilical vein endothelial cells were treated with or without specific inhibitors of NADPH oxidase (100 μg/mL APO), cytochrome P-450 (5 μmol/L SKF), or xanthine oxidase (30 μmol/L AL) for 24 hours and stimulated with TNF (100 U/mL) for 12 hours or H$_2$O$_2$ (50 μmol/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.

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$^5$ cells per 30 minutes, suggesting a possible role for O$_2^-$ in NF-κB-mediated VCAM-1 induction by TNF. SKF525a and Apocynin interfered with the absorbance of ferricytochrome c, probably because of NADPH-independent reduction of cytochrome c, its effect on O$_2^-$ generation cannot be assessed in this system.

H$_2$O$_2$ activates NF-κB in T cells but not in monocytes or a cell-free system. After membrane diffusion, H$_2$O$_2$ is converted into O$_2^-$ or hydroxyl radicals (OH·) via the Haber-Weiss reaction. We tested whether H$_2$O$_2$ stimulates NF-κB–dependent VCAM-1 induction. Treatment of HUVECs with H$_2$O$_2$ was performed at 50 μmol/L for 6 hours. These conditions did not affect cell viability. Although expression of ICAM-1 and PECAM-1 was not altered, H$_2$O$_2$, slightly but significantly induced VCAM-1 sMFI to 26±3 channels (P<.05, n=3), indicating that ROIs alone are not sufficient for VCAM-1 induction by TNF.

Radicals are known to be generated by NADPH oxidase, xanthine oxidase, or cytochrome P-450–dependent oxidase in endothelial cells. Hence, we examined the contribution of these enzymes to VCAM-1 induction. The plant phenol apocynin, a selective inhibitor of neutrophil O$_2^-$ release, partially inhibited VCAM-1 induction by TNF (Table 4), best at 100 μg/mL (Fig 6), supporting the idea that ROIs produced by activated NADPH oxidase are not exclusively responsible for mediation of NF-κB induction. Although the xanthin oxidase inhibitor allopurinol had no effect, pretreatment with the specific cytochrome P-450 inhibitor SKF525a (5 μmol/L) resulted in reduced NF-κB mobilization (Fig 5) and VCAM-1 induction by TNF (Fig 6). Apocynin (50 μg/mL) and SKF525a (2.5 μmol/L) showed additive effects on VCAM-1 induction (Table 4), suggesting distinct mechanisms of action rather than mutual interference. This indicates that NF-κB–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 counter receptor for ICAM-1. Stimulation of HUVECs with

![Fig 7. Bar graph shows effect of pyrrolidine dithiocarbamate (PDTC) on vascular cell adhesion molecule-1 (VCAM-1)–dependent U937 cell adhesion to tumor necrosis factor–α (TNF)–stimulated human umbilical vein endothelial cells. Cells were pretreated without (closed column) or with PDTC at indicated concentrations, apocynin (100 μg/mL, APO) and SKF525a (5 μmol/L, SKF) for 24 hours, or anti–VCAM-1 monoclonal antibody 1G11 (100 μg/mL, 1G11) for 24 hours, and stimulated with TNF at indicated concentrations for 24 hours. Adhesion of fluorescence-labeled U937 cells was determined by comparison to a standard curve. Data are expressed as percent adhesion of cells added and represent mean±SD of four separate experiments performed in duplicate. *P<.01 vs TNF (100 μg/mL).](http://atvb.ahajournals.org/doi/pdf/10.1161/01.ATV.14.10.1670)
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.\(^1\) The major contribution of VCAM-1 to U937 cell adhesion is also evident from our finding that basal adhesion to unstimulated HUVECs was negligible decreased, whereas stimulation of HUVECs with interferon gamma (100 U/mL) induced VCAM-1 but not ICAM-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.\(^2\) (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 \(\mu\)mol/L PDTC for 24 hours caused marked inhibition of TNF-induced adhesion (75%). SKF525a (5 \(\mu\)mol/L) or apocynin (100 \(\mu\)g/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-\(\kappa\)B mobilization\(^2\) 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-\(\kappa\)B mobilization and subsequent VCAM-1 transcription.

**Discussion**

\(\kappa\)B-like elements are present in regulatory sequences of VCAM-1 and ICAM-1 genes.\(^3\)\(^,\)\(^4\)\(^1\)\(^7\)\(^,\)\(^1\)\(^9\) To analyze the role of NF-\(\kappa\)B 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-\(\kappa\)B 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.\(^2\)\(^2\)\(^3\)\(^4\)\(^\) In HeLa cells, PDTC rather induced AP-1 transactivation and DNA binding.\(^3\)\(^1\) In HUVECs, PDTC almost completely blocked TNF-stimulated NF-\(\kappa\)B 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-\(\kappa\)B–like regulatory factors is responsible for VCAM-1 but not ICAM-1 induction by TNF in HUVECs. Consistent with these results, both \(\kappa\)B sites in the VCAM-1 enhancer are required for optimal gene expression.\(^1\)\(^4\) The suppressive effect of PDTC on VCAM-1 induction by TNF reveals a biphasic dose-response curve with relieved inhibition at 300 \(\mu\)mol/L, which exactly resembles the biphasic dose–response relation observed for suppression of NF-\(\kappa\)B mobilization by PDTC in T cells.\(^2\)\(^2\) A biphasic dose dependence may also reflect oxidative properties of thiols at concentrations greater than 100 \(\mu\)mol/L.\(^3\)\(^6\) Based on this characteristic dose response, interference of PDTC with TNF receptor or DNA binding activity of NF-\(\kappa\)B and disintegration of cells appear unlikely, indicating a specific action of PDTC on NF-\(\kappa\)B–mediated VCAM-1 induction. At high concentrations, p65 alone stimulates the VCAM-1 promoter in HUVECs; at low concentrations it acts in concert with p50.\(^1\) Selectivity may be determined by dimeric association of specific NF-\(\kappa\)B subunits, allowing differential regulation of genes containing \(\kappa\)B sites.\(^7\) Elements flanking \(\kappa\)B sites may interact with activated NF-\(\kappa\)B to modulate initiation of transcription from distinct promoters. This may explain why NF-\(\kappa\)B appears to be essential for VCAM-1 but not sufficient for ICAM-1 and ELAM-1 induction.\(^1\) 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.\(^3\)\(^8\)

Cultured arterial endothelial cells have been reported to express VCAM-1 and increased ICAM-1 after treatment with lysophosphatidylcholine, a component of atherogenic lipoproteins.\(^3\) 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,\(^1\)\(^7\)\(^2\)\(^1\) this implies that PDTC rather acts by interfering with radicals. To further analyze the molecular mechanisms of NF-\(\kappa\)B–mediated VCAM-1 induction by TNF, we focused on a recently suggested role of ROS in the mediation of NF-\(\kappa\)B activation.\(^2\)\(^1\)\(^4\)\(^0\) Importantly, we demonstrated that HUVECs produced \(\mathrm{O}_2^-\) in response to TNF, indicating a possible role of \(\mathrm{O}_2^-\) in mediating NF-\(\kappa\)B 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 \(\mathrm{H}_2\mathrm{O}_2\) to \(\mathrm{OH}^-\) by the Fenton reaction, \(\mathrm{OH}^-\) was proposed to be mainly responsible for NF-\(\kappa\)B activation. However, \(\mathrm{H}_2\mathrm{O}_2\) 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.\(^3\) Consistent with our results, the PDTC-induced inhibition of NF-\(\kappa\)B mobilization in monocytes was not reversible by Co\(^2+\) or Mn\(^2+\) cations, which block the suppression of NF-\(\kappa\)B mobilization by the metal chelator orthophenanthroline.\(^3\)\(^0\)

The slight enhancement of HUVEC VCAM-1 expression by \(\mathrm{H}_2\mathrm{O}_2\) suggests that besides \(\mathrm{O}_2^-\), other mediators have to contribute to maximal VCAM-1 induction. In monocytes, elements of NADPH oxidase appear not to be required for NF-\(\kappa\)B activation.\(^3\)\(^8\) Since HUVECs can generate radicals by various enzymes, such as NADPH oxidase,\(^3\) xanthine oxidase,\(^3\) or cytochrome P-450–dependent oxidase,\(^3\)\(^4\) we assessed their conceivable involvement in VCAM-1 induction by TNF. In neutro-
phils stimulated to release ROIs and peroxidase, the plant phenol apocynin is activated to metabolites that prevent assembly of functional, \( O_2^\cdot \)-generating NADPH oxidase in the membrane by conjugation to thiol groups. \(^{30}\) Inhibition by apocynin has implicated NADPH oxidase in lipopolysaccharide-induced ELAM-1 mRNA expression in HUVECs. \(^{41}\) 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), \(^{34}\) which promotes U937 adhesion to HUVECs. \(^{42}\) Moreover, oxidized low-density lipoprotein may influence gene expression by causing oxidative stress and NF-\( \kappa \)B activation. \(^{43}\) SKF525a, which prevented 14,15-EET synthesis in HUVECs, \(^{44}\) 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. \(^{42}\) Since NF-\( \kappa \)B activation by TNF was found to be controlled by ROIs derived from the respiratory chain in fibrosarcoma cells, \(^{45}\) as demonstrated by depletion of mitochondrial electron transport, SKF525a may act by interference with electron entry to mitochondrial ubiquinone pools. \(^{44}\) In T cells, esterified vitamin E derivatives, but not vitamin E, inhibit TNF-induced NF-\( \kappa \)B mobilization, implying that oxidative metabolism processes required for cell signaling are localized in compartments accessible only to vitamin E derivatives. \(^{45}\) 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, \(^{46}\) ROIs have been proposed to selectively activate phosphorylation \(^{47}\) via a redox-regulated tyrosine kinase \(^{48}\) or to induce I\( \kappa \)B degradation by a cytoplasmic protease. \(^{49}\) In accordance with our results with PDTC, neither \( \mathrm{H}_2\mathrm{O}_2 \) 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 (CD16/CD18). ICAM-1, which may account for polymorphonuclear neutrophil adhesion and migration, \(^{7}\) 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. \(^{2}\) 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, \(^{29}\) emphasizing the importance of VCAM-1 for monocyte adhesion. \(^{6}\) 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. \(^{49}\) Immunohistochemical staining revealed VCAM-1 expression in aortic endothelium covering foam cell lesions or extending beyond their edges in hyperlipidemic mice, \(^{50}\) in areas of neovascularization and inflammatory infiltration of human coronary atherosclerotic plaques, \(^{51}\) after aortic balloon injury in rabbit vascular cells, \(^{52}\) and in allograft biopsies. \(^{53}\) Oxidative stress and subsequent NF-\( \kappa \)B activation have been implicated in the induction of endothelial genes associated with the initiation of atherosclerosis. \(^{54}\) 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). \(^{55}\)

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