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Research Article

Pycnogenol inhibits tumor necrosis factor-α-induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells

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Abstract. The transcriptional regulatory protein nuclear factor kappa B (NF-κB) participates in the control of gene expression of many modulators of inflammatory and immune responses, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). The heightened expression of these adhesion molecules has been reported to play a critical role in atherosclerosis, inflammation, ischemic vascular disorders, diabetes, and cancer metastasis. In the present study, we investigated the effect of pycnogenol, an antioxidant phytochemical, on the activation of NF-κB and the induction of VCAM-1 and ICAM-1 in tumor necrosis factor (TNF)-α-treated human umbilical vein endothelial cells (HUVECs). Gel-shift analysis of HUVEC demonstrated that pretreatment with pycnogenol exhibited a concentration-dependent suppression of TNF-α-induced activation of NF-κB. Induction of VCAM-1 and ICAM-1 surface expression by TNF-α was dose-dependently reduced by pycnogenol. TNF-α significantly increased the release of superoxide anion and hydrogen peroxide from HUVECs. Pycnogenol dose-dependently inhibited their release. The ability of pycnogenol to inhibit NF-κB activation and VCAM-1 and ICAM-1 expression suggests that this phytochemical may play an important role in halting or preventing the atherogenic process.

Key words. Pycnogenol; nuclear factor kappa B; adhesion molecule; antioxidant; human umbilical vein endothelial cell.

Introduction

Early features in the development of atherosclerosis include enhanced expression of endothelial vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [1, 2]. The heightened expression of these adhesion molecules has been reported to play a critical role in atherosclerosis, inflammation, ischemic vascular disorders, diabetes, and cancer metastasis [3–5]. Nuclear factor kappa B (NF-κB) is a nuclear protein of the Rel gene family involved in regulation of numerous genes encoding proteins, including VCAM-1 and ICAM-1 [6, 7]. The promoter region of the genes for these adhesion molecules contains NF-κB-binding sites essential for its expression [7]. The role of NF-κB in the activation of adhesion molecule expression has been studied using human umbilical vein endothelial cells (HUVECs) [8, 9]. Analyses of the promoter regions of VCAM-1 and ICAM-1 have indicated that NF-κB plays a pivotal role in cytokine-induced gene expression [10, 11].

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The DNA-binding subunits of NF-κB comprise six members in mammals: p50, p65 (Rel A), c-Rel, v-Rel, p52, and Rel B [12]. Existing in the cytoplasm as an inactive form, NF-κB is stabilized by an inhibitory subunit IκB [13]. A variety of signals such as hydrogen peroxide (H_2O_2), tumor necrosis factor (TNF)-α and TNF-β, interleukin (IL)-1 and IL-2, hypoxia, virus, protein synthesis inhibitors, and phorbol esters induce degradation of IκB, allowing free NF-κB dimers to migrate to the nucleus. The free NF-κB dimers then bind to κB recognition elements in a variety of genes, including elements in VCAM-1 and ICAM-1 promoters, and activate gene expression in conjunction with other transcription factors [14].

TNF-α stimulates the production of reactive oxygen species (ROS) in a variety of cell types [15, 16] and ROS can serve as signals facilitating NF-κB activation [17]. Studies have shown that antioxidants inhibited cytokine-induced VCAM-1 and ICAM-1 expression in cultured HUVECs [11, 18]. These agents have been assumed to inhibit adhesion molecule expression primarily by blocking NF-κB activation.

Pycnogenol is a proprietary mixture of water-soluble bioflavonoids extracted from the bark of French maritime pine (*Pinus maritima*). It mainly includes phenolic compounds, broadly divided into monomers (catechin, epicatechin, and taxifolin) and condensed flavonoids (classified as procyanidins/proanthocyanidins). About 85% of the compounds in pycnogenol are identified as procyanidins. Of these procyanidins, about 60% are dimers and trimers, 20% are oligomers and phenolic acids such as gallic acid, caffeic acid, and ferulic acid. The whole extract or individual fraction has been shown to have strong antioxidant capacity in in vitro cultured cells, perfused organs, and in vivo models [19]. We have demonstrated that pycnogenol can protect vascular endothelial cells from injury induced by an organic oxidant, *t*-butyl hydroperoxide [20]. It can increase the levels of intracellular glutathione and enhance the activities of antioxidant enzymes [21]. Pycnogeneol also inhibited the macrophage oxidative burst, lipoprotein oxidation, and hydroxyl-radical-induced DNA damage [22]. More recently, we have shown that pycnogenol enhances immune and hemopoietic functions and counters learning impairment and memory deficit in senescence-accelerated mice [23, 24]. In this study, we determined the effects of pycnogenol on TNF-α-induced activation of NF-κB and expression of VCAM-1 and ICAM-1.

Materials and methods

Materials. Pycnogenol was provided by Henkel Corporation, La Grange, Ill. 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes, Eugene, Ore. Hydroethidine (HE) was from Polysciences, Warrington, Pa. TNF-α, NF-κB-specific oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), nonspecific activator protein-1 (AP1) oligonucleotide (5′-CGC TTG ATG AGT CAG CCG GAA-3′) and T4 polynucleotide kinase were obtained from Promega (Madison, Wisc.). Anti-human ICAM-1 (BBIG-I1) and anti-human VCAM-1 (BBIG-V1) monoclonal antibody were purchased from R & D Systems, Minneapolis, Minn. Anti-goat IgG peroxidase conjugate was obtained from Boehringer Mannheim, Indianapolis, Ind. Poly(dI-dC) was supplied by Amersham Pharmacia Biotech, Piscataway, N.J. [γ-^{32}P]ATP was from ICN Pharmaceuticals, Costa Mesa, Calif. Specific antibodies to NF-κB subunits p50 and p65 were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif. Endothelial cell growth supplement (ECGS) was purchased from Fisher Scientific, Tustin, Calif. Trypsin-EDTA solution and penicillin-streptomycin solution were supplied by Mediatech, Herndon, Va. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories, Logan, Utah. Coomassie protein assay reagent was from Pierce (Rockford, Ill). F12K medium, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), antipain, leupeptin, Nonidet p-40, bovine serum albumin (BSA), phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), ethylenediamine tetraacetic acid (EDTA), and o-phenylenediaine dihydrochloride (OPD) were purchased from Sigma (St. Louis, Mo.).

Cell culture. HUVECs were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). Cells were subcultured in F12K medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml heparin, 40 μg/ml ECGS, and 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. They were used within the first five passages. HUVECs were harvested from 75-cm^2 culture flasks by trypsinization, counted, and seeded in 96-well and 48-well plates (Nunc) or 100 × 20 mm tissue culture dishes (Falcon 3003) at a density of 2.5×10^4 or 5×10^4 cells/well, respectively, or 3×10^6 cells/dish. After growing to confluence, cells grown in 96-well plates were used for enzyme-linked immunosorbent assay (ELISA) and cells in 24-well plates were used to determine superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Cells grown in dishes were used for determination of NF-κB activation induced by TNF-α. The viability of cells used throughout the experiments was always greater than 90% as determined by trypan blue exclusion.

Experimental design. The effect of TNF-α on NF-κB activation was determined first. Different concentrations of TNF-α in HBSS were added to dishes, incubated at 37 °C and 5% CO_2 for 2 h, followed by nuclear extraction. An electrophoretic mobility shift assay (EMSA) was then performed. To ascertain that the

activated DNA-binding protein was NF-κB specific, a binding competition assay was performed. Fifty- and tenfold unlabeled NF-κB-specific oligonucleotide and fiftyfold nonspecific AP1 oligonucleotide were individually mixed with the nuclear extract 15 min before adding the ^{32}P-labeled NF-κB probe. To identify the components of NF-κB complexes induced by TNF-α, peptide-specific rabbit antisera to p50 and p65, and normal rabbit serum were used in the supershift assay. To determine the effects of pycnogenol on NF-κB activation induced by TNF-α, HUVECs were preincubated with various concentrations of pycnogenol. After the removal of pycnogenol, cells were washed with HBSS and exposed to TNF-α for 2 h, followed by nuclear extraction and EMSA. Experiments were carried out to determine the effects of pycnogenol on VCAM-1 and ICAM-1 expression induced by TNF-α. The expression of adhesion molecules was measured by ELISA. H_2O_2 and O_2^- were also determined by a fluorometric assay.

Nuclear extracts. Nuclear extracts were prepared as previously described [25]. Cells were harvested by centrifugation for 10 min at 1200 rpm (100 × g) at 4 °C, resuspended in 1 ml of ice-cold PBS, and centrifuged again for 15 s at 14,000 rpm (10,000 × g) at 4 °C. Lysing buffer [0.8 ml of 10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 10 mg/ml antipain and 10 mg/ml of leupeptin] was added to the pellet, vortexed for 15 s, and kept on ice for 15 min. Nonidet p-40 solution (10%, 25 μl) was added and the pellet was vigorously mixed for 15 s and centrifuged for 30 s at 14,000 rpm at 4 °C. Pelleted nuclei were resuspended in 100 μl of extraction buffer [50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% (v/v) glycerol]. Following gentle mixing for 20 min, the tubes were spun for 10 min at 14,000 rpm, after which the supernatant containing the nuclear proteins was transferred to another tube and stored at − 80 °C. Protein concentration was determined using the Coomassie protein assay reagent.

Electrophoretic mobility shift assay. NF-κB-specific oligonucleotide was end-labeled with [γ-^{32}P]ATP (7,000 Ci/mM) and purified using a NAP-5 column (Pharmacia Biotech). An EMSA was performed according to the method of Wei et al. [25]. Ten micrograms of nuclear protein, 2 μl of binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl and 0.25 mg/ml of poly(dI-dC)], 1 μl of ^{32}P-labeled oligonucleotide and 1 μl of loading buffer (250 mM Tris-HCl, 0.2% bromophenol blue, and 40% glycerol) were incubated for 20 min at 25 °C. In competition assays, fiftyfold and tenfold excess unlabeled oligonucleotide competitors were added 15 min before addition of radiolabeled probes. DNA-protein complexes were separated from the unbound DNA probe by electrophoresis through a native 6% polyacrylamide gel in 0.5 × TBE (Tris-borate-EDTA buffer, pH 8.0). Gels were vacuum dried and autoradiographed. The relative intensity of NF-κB bands was quantified by densitometry scanning of autoradiographs using the Bio Image Whole Band Analyzer, version 3.0 (Millipore, Ann Arbor, Mich.). For the supershift assay, nuclear extracts were incubated with 2 μg of anti-p50 and anti-p65 for 30 min at room temperature. These mixtures were then subjected to EMSA.

Enzyme-linked immunosorbent assay. HUVECs in F12K medium were cultured in 96-well plates. After growing to confluence, cells were then exposed to TNF-α (0, 5, 10, 20, 40, and 60 ng/ml) and incubated for 6 h. Cells were washed twice and blocked with 5% nonfat dry milk in PBS for 10 min. The primary antibodies (0.5 μg/ml anti-VCAM-1 or 0.4 μg/ml anti-ICAM-1 in 5% BSA/FBS solution) were added to each well and incubated for 45 min at 37 °C. Cells were then washed three times and incubated for 45 min with anti-goat IgG peroxidase conjugate diluted 1:1000 in PBS with 5% FBS. After washing three times, the substrate (o-phenylenediamine dihydrochloride) was added for 1 h and the reaction was quenched with 8 N sulfuric acid. Optical density was determined at 450 nm using an automated microplate reader (Bio-Rad, Hercules, Calif.).

Assays for superoxide anion. The level of O_2^- was determined using HE, the reduced form of the fluorescent compound ethidine bromide (EB), as a probe [21]. Confluent monolayers of HUVECs were incubated with pycnogenol in complete culture medium at 37 °C for 20 h. After removal of pycnogenol, cells were treated with 60 ng/ml TNF-α for 2 h. HE (125 μM, 0.25 ml) was added to 24-well plates and incubated for 30 min. Fluorescence intensity (relative fluorescence units, RFU) was measured using 540-nm excitation and 620-nm emission in a 7620 Microplate Fluorometer (Cambridge Technology, Watertown, Mass.).

Assays for hydrogen peroxide. The level of H_2O_2 was determined by a fluorometric assay using DCFH-DA as a probe [21]. HUVECs were preincubated with pycnogenol followed by treatment with 60 ng/ml of TNF-α for 2 h; 10 μl of 0.5 mM DCFH-DA (dissolved in N,N-dimethylformamide) was then added to wells. The fluorescence intensity (RFU) was measured 30 min later at 485-nm excitation and 530-nm emission using a 7620 Microplate Fluorometer.

Statistical analyses. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test for significant differences. Statistical significance was defined as $P < 0.05$. All statistical procedures were performed with Statgraphics software version 5.0 (STSC, Rockville, Md.).

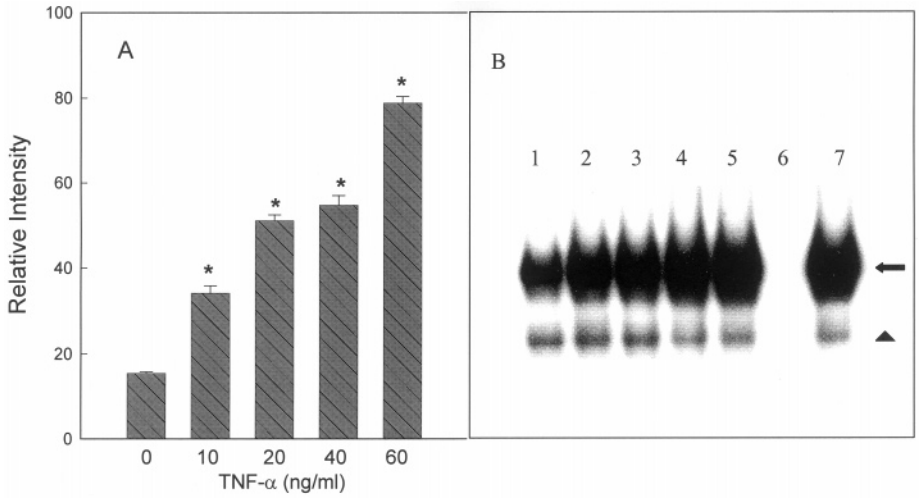

Figure 1. NF-κB activation induced by TNF-α. (*A*) Nuclear extracts from cells treated with 0, 10, 20, 40, and 60 ng/ml of TNF-α were used. Samples were assayed for NF-κB-binding activity by EMSA. TNF-α induced a concentration-dependent activation of NF-κB. Values are expressed as relative intensity of radioactivity. Data represent means ± SE of three experiments. Asterisks denote a significant difference from control without TNF-α ($P < 0.05$). (*B*) Lanes 1–5 = nuclear extracts from cells treated with 0, 10, 20, 40, and 60 ng/ml of TNF-α, respectively; lane 6 = nuclear extract from 60 ng/ml of TNF-α-treated endothelial cells incubated with 50-fold unlabeled NF-κB-specific oligonucleotide before adding ^{32}P-labeled probe; lane 7 = nuclear extract from 60 ng/ml of TNF-α-treated endothelial cells incubated with 50-fold unlabeled NF-κB nonspecific oligonucleotide before adding ^{32}P-labeled probe. The arrow indicates p50/p65 heterodimer NF-κB. The triangle indicates unidentified NF-κB.

Results

To determine the appropriate concentration of TNF-α for NF-κB activation, HUVECs were incubated with 10–60 ng/ml of TNF-α for 2 h. Nuclear extracts were prepared and analyzed for the specific DNA binding of NF-κB using EMSA. NF-κB activation from HUVECs yielded two bands (fig. 1B). The upper band showed a concentration-dependent increase at the doses of 10–60 ng/ml of TNF-α as quantified by densitometry scanning (fig. 1A). Cell viability was not decreased at 10–60 ng/ml of TNF-α. TNF-α at 60 ng/ml was chosen for subsequent experiments. NF-κB bands induced by TNF-α were identified by specific and nonspecific competitors, and antibody supershift assay. A faint NF-κB band was visible in nuclear extract with tenfold unlabeled κB oligonucleotide (fig. 2, lane 3); however, both bands of nuclear extract were competitively inhibited by a 50-fold excess of unlabeled κB oligonucleotide (fig. 2, lane 4), but not by the nonspecific oligonucleotide (fig. 2, lane 5). Nuclear extracts from HUVECs were incubated with 2 μg of normal rabbit serum or antisera p50 and p65. As shown in figure 2, normal rabbit serum did not affect complex formation (lane 8). Antibody specific for the p50 or p65 subunit of NF-κB supershifted the upper band but not the lower band, indicating that

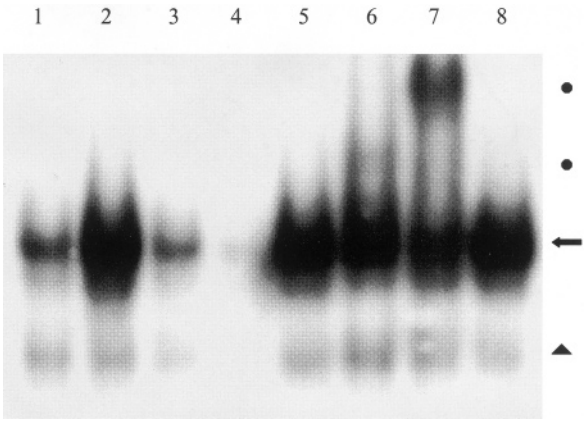

Figure 2. The TNF-α-induced NF-κB complex comprises p50 and p65. Lane 1 = untreated cells; lane 2 = nuclear extract from cells treated with 60 ng/ml TNF-α; lanes 3, 4 = nuclear extracts from 60 ng/ml TNF-α-treated endothelial cells incubated with 10- and 50-fold unlabeled NF-κB-specific oligonucleotide, respectively, before adding ^{32}P-labeled probe; lane 5 = nuclear extract from 60 ng/ml TNF-α-treated endothelial cells incubated with 50-fold unlabeled NF-κB nonspecific oligonucleotide before adding ^{32}P-labeled probe; lanes 6–8 = nuclear extracts from TNF-α-treated cells with 2 μg of anti-p50, anti-p65, and normal rabbit serum, respectively. Circles indicate the bands shifted by the antisera. The arrow indicates p50/p65 heterodimer NF-κB. The triangle indicates unidentified NF-κB.

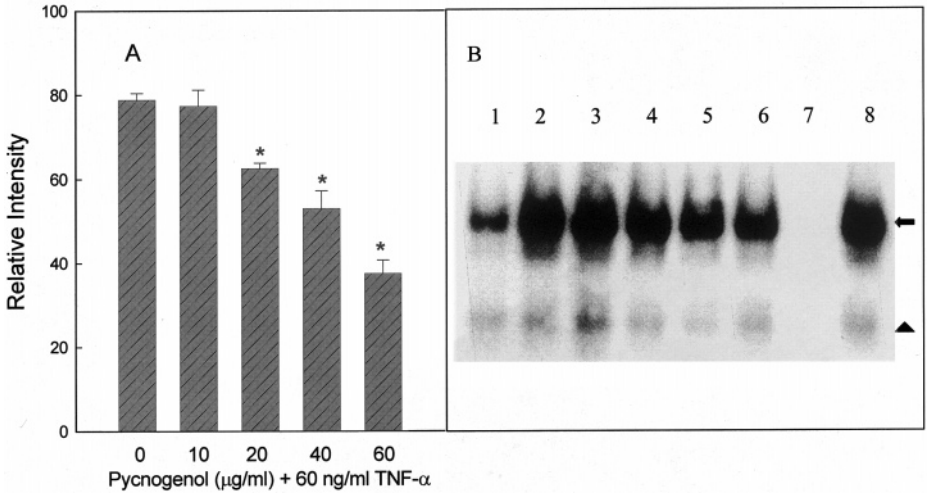

Figure 3. The effect of pycnogenol on NF-κB activated by TNF-α. (*A*) Cells were preincubated with 0, 10, 20, 40, and 60 μg/ml of pycnogenol for 20 h, followed by treatment with 60 ng/ml TNF-α for 2 h. Nuclear extracts were prepared and analyzed by EMSA. Pycnogenol showed a concentration-dependent inhibition of TNF-α-induced NF-κB activation. Values are expressed as relative intensity of radioactivity. Data represent means ± SE of three experiments. Asterisks denote a significant difference compared with extract without pycnogenol ($P < 0.05$). (*B*) Pycnogenol exhibits a concentration-dependent inhibition of NF-κB activated by TNF-α. Lane 1 = cells with no pycnogenol or TNF-α treatment; lane 2 = nuclear extract from 60 ng/ml of TNF-α-treated cells; lanes 3–6 = cells were pretreated with 10, 20, 40, and 60 μg/ml of pycnogenol, respectively, followed by 60 ng/ml of TNF-α; lane 7 = nuclear extract from ng/ml TNF-α-treated endothelial cells incubated with 50-fold unlabeled NF-κB-specific oligonucleotide before adding ^{32}P-labeled probe; lane 8 = nuclear extract from 60 ng/ml of TNF-α-treated endothelial cells incubated with 50-fold unlabeled NF-κB nonspecific oligonucleotide before adding ^{32}P-labeled probe. The arrow indicates p50/p65 heterodimer NF-κB. The triangle indicates unidentified NF-κB.

TNF-α induced p65/p50 of NF-κB. The lower band was unidentified but could represent the monomeric p52 component of NF-κB.

To study the effect of pycnogenol on NF-κB activation, HUVECs were preincubated with 0, 10, 20, 40, and 60 μg/ml of pycnogenol followed by treatment with 60 ng/ml of TNF-α. Induction of NF-κB was decreased by increasing concentrations of pycnogenol (fig. 3). Pycnogenol at 10, 20, 40, and 60 μg/ml inhibited NF-κB activation by 16.08, 25.74, 49.97 and 59.84%, respectively.

The effects of pycnogenol on the TNF-dependent expression of ICAM-1 and VCAM-1 in HUVECs were examined using ELISA. As shown in figure 4A, VCAM-1 expression was barely detectable in unstimulated cells but was markedly induced after stimulation with 60 ng/ml TNF-α for 6 h. Pretreatment of HUVECs with pycnogenol for 20 h before stimulation with TNF-α markedly suppressed TNF-α-induced VCAM-1 surface expression. At 60 μg/ml of pycnogenol, the induction of cell surface VCAM-1 by TNF-α was inhibited by 76%. The inhibitory effect of pycnogenol could be noted even at 10 μg/ml. In contrast, ICAM-1 was constitutively expressed by untreated HUVECs and significantly upregulated by TNF-α (fig. 4B). In the presence of 60 μg/ml pycnogenol, the induced expression of cell surface ICAM-1 was inhibited by 85%, with significant inhibition first noted at 20 μg/ml of pycnogenol.

The effect of pycnogenol on TNF-α-induced O_2^- and H_2O_2 release in HUVECs was also studied. Stimulation of HUVECs with 60 ng/ml TNF-α resulted in a 4.7-fold increase of O_2^- and 4.4-fold increase of H_2O_2 release compared to the control without TNF-α treatment (from baseline of 131 to 618 RFU for O_2^-, and 120 to 521 RFU for H_2O_2). Preincubation with pycnogenol significantly inhibited O_2^- and H_2O_2 release, and the inhibition was concentration dependent (fig. 5). We tested the toxicity of pycnogenol and TNF-α to HUVECs by trypan blue exclusion. Cell viability was always greater than 95% after incubation with TNF-α for 2 or 6 h following preincubation with pycnogenol for 20 h.

Discussion

TNF-α has been shown to activate NF-κB in various types of cells [26, 27]. The data presented in this study show that TNF-α treatment of HUVECs increases the activation of NF-κB, especially p65. A nuclear extract from HUVECs has two bands. The binding of the two bands was specific, as both were competitively inhibited

by an excess of unlabeled κB oligonucleotide. In endothelial cells, NF-κB has been described to consist predominantly of the p50 and p65 heterodimer [2]. Our supershift assay results confirm that the upper band is composed of p50 and p65 subunits in HUVECs.

In this study, the gel retardation analyses demonstrate that pycnogenol can significantly inhibit the activation of NF-κB induced by TNF-α. The concentration of pycnogenol used in this study did not affect cell viability. Thus the decreased level of NF-κB is not due to cell death. Previous studies have shown that NF-κB/Rel proteins are involved in the cytokine-induced upregulation of VCAM-1 and ICAM-1 in endothelial cells [7, 8, 28]. The activation of the NF-κB heterodimer by TNF-α in HUVECs may lead to activation of its target genes. Indeed, the data presented in this study show that

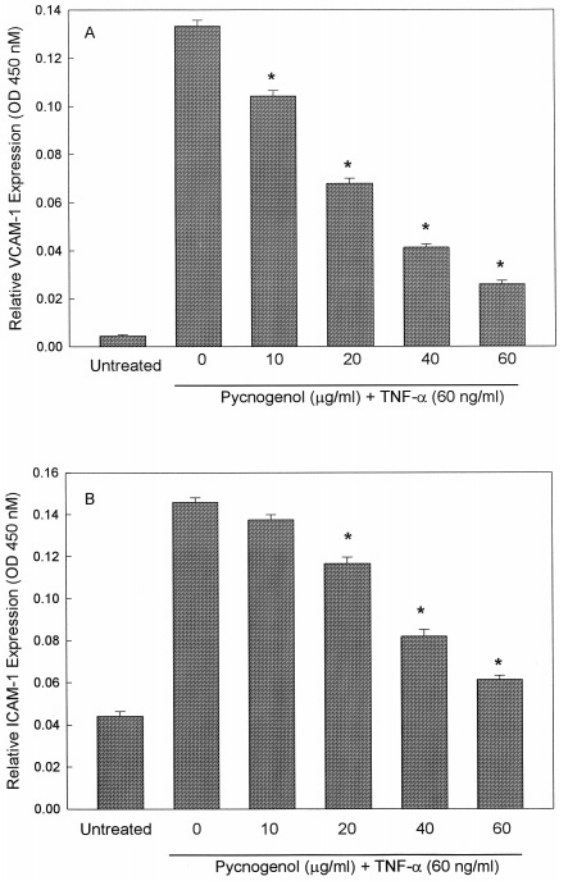

Figure 4. Effects of pycnogenol on TNF-α-induced VCAM-1 (*A*) and ICAM-1 (*B*) expression. Confluent HUVEC monolayers were cultured in a 96-well plate and treated with various concentrations of pycnogenol for 20 h before addition of 60 ng/ml of TNF-α for 6 h. Cell surface expression was measured by ELISA. Samples were performed in triplicate. Results are expressed as means ± SE of optical density. Asterisks denote significant difference ($P < 0.05$) compared to control without pycnogenol treatment.

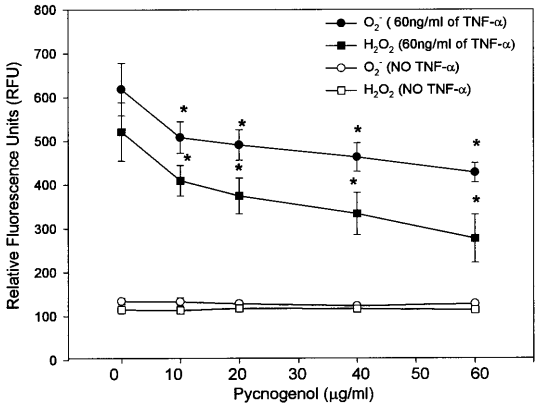

Figure 5. Effects of pycnogenol on TNF-induced O_2^- and H_2O_2 levels. Confluent monolayers of HUVECs were incubated with 0, 10, 20, 40, and 60 μg/ml of pycnogenol in complete culture medium at 37 °C for 20 h. After washing three times with HBSS to remove pycnogenol, cells were treated with 60 ng/ml TNF-α for 2 h. For the O_2^- assay, HE (125 μM, 0.25 ml) was added to the cells and incubated for 30 min. EB fluorescence was recorded. For H_2O_2, DCFH-DA (0.5 mM) was added to the wells. After 30 min incubation at 37 °C, DCF fluorescence was measured. Asterisks denote a significant difference ($P < 0.05$) compared to control without pycnogenol treatment.

TNF-α induced the expression of both VCAM-1 and ICAM-1 in HUVECs markedly (fig. 4). These adhesion molecules are involved in vascular injury, acute and chronic airway obstruction, renal inflammation, and tumor cell adhesion to endothelium [4, 5, 29]. NF-κB has been shown to be essential for the gene expression of these adhesion molecules; therefore, inhibition of this transcription factor is expected to inhibit the induction of NF-κB-dependent genes. Protease inhibitors or antioxidants have been shown to inhibit cytokine-induced VCAM-1 gene expression by blocking the degradation of IκB-α and subsequent nuclear translocation of NF-κB/Rel proteins in endothelial cells [11, 18, 30]. However, NF-κB-independent induction of VCAM-1 has also been demonstrated in endothelial cells. A proteosome inhibitor that blocks IL-1-induced VCAM-1 and ICAM-1 gene expression in endothelial cells without inhibiting NF-κB activation has been reported [31]. In our study, pycnogenol inhibited TNF-α-induced expression of VCAM-1 and ICAM-1, as well as NF-κB activation.

Early work implicated a role for ROS in TNF-α-mediated NF-κB activation and adhesion molecule expression, conclusion based largely on ROS generation induced by TNF-α and suppression of cytokine-induced NF-κB activation and VCAM-1 and ICAM-1 expression by antioxidants [26, 27, 32, 33]. However, the molecular basis for this regulation is largely unknown [34]. The effects of antioxidants on NF-κB activation

and generation of ROS in response to NF-κB inducer are stimulus and cell specific [17]. Oxidative stress has recently been suggested to be facilitatory rather than causal in TNF-α-induced NF-κB activation. Lipid peroxide may regulate TNF-α-mediated NF-κB activation [17]. Our results show that HUVECs produced O_2^- and H_2O_2 in response to TNF-α, indicating a possible role for ROS in mediating NF-κB mobilization and subsequent VCAM-1 and ICAM-1 induction. Pycnogenol has been recognized as a potent antioxidant and free radical scavenger. Its biological activity and mechanisms of action have been extensively discussed in a recent review [19]. The molecular bases of pycnogenol activity are manyfold, but appear to depend mainly on its capacity to scavenge reactive oxygen and reactive nitrogen species efficiently. It can also bind to proteins, thereby affecting both their structural and functional characteristics. Pycnogenol participates in the cellular antioxidant network and affects the expression of those genes that are regulated by cell redox status [19]. We previously demonstrated that pycnogenol protected vascular endothelial cells from oxidant injury because of its antilipid peroxidation activity [20]. It enhanced the intracellular glutathione level and antioxidant enzyme activities and inhibited lipoprotein oxidation and hydroxyl-radical-induced DNA damage [21, 22]. An increase in intracellular levels of glutathione or thiol, and overexpression of antioxidant enzymes have been shown to inhibit TNF-mediated NF-κB activation [35–37]. The current study shows that pycnogenol can significantly decrease the levels of O_2^- and H_2O_2 induced by TNF-α in HUVECs. Therefore, the mechanism of blocking TNF-α-induced NF-κB activation and VCAM-1 and ICAM-1 expression by pycnogenol may be associated with its strong antioxidant activity. Its antilipid peroxidation effect may be one of the mechanisms by which pycnogenol inhibits NF-κB activation in response to TNF-α, since a role of lipid peroxidation rather than H_2O_2 in some pathways to NF-κB has been suggested [17]. Evidence is accumulating that NF-κB and its inhibitors may play a key role in regulating vascular pathophysiology. Functional NF-κB elements can be found in many genes whose expression is increased in vascular cells at sites of inflammation [2]. The role of ICAM-1 and VCAM-1 in atherosclerotic lesions, inflammation, adhesion of tumor cells to endothelial cells, and graft-versus-host diseases has been established [3–5]. The results shown here clearly indicate that pycnogenol can block TNF-α-stimulated NF-κB activation and inhibit VCAM-1 and ICAM-1 induction. The suppressive effect of pycnogenol on NF-κB activation and on these adhesion proteins implies that this phytochemical would be useful for either the prevention or the treatment of atherosclerosis, inflammation, and tumor metastasis.

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