Inhibition of Tumor Necrosis Factor Induced Human Aortic Endothelial Cell Adhesion Molecule Gene Expression by an Alkoxybenzo[b]thiophene-2-carboxamide

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Summary: The effects of a novel anti-inflammatory agent, 5-methoxy-3-(1-methyl-ethoxy)benzo[b]thiophene-2-carboxamide-1-oxide (PD 144795) on adhesion molecule expression in tumor necrosis factor (TNF) stimulated human aortic endothelial cells (HAEC) were examined. PD 144795 treatment markedly inhibited the TNF-induced cell expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) protein and m-RNA. Gel shift assays using nuclear extracts from HAEC treated with PD 144795 failed to show a decrease in the activation of NFkB by this compound, whereas pyrrolidine dithiocarbamate (PDTC), an antioxidant, markedly inhibited the activation of this transcription factor. Thus, PD 144795 inhibits agonist-stimulated VCAM-1 and ICAM-1 expression likely via an NFkB independent mechanism, distinct from that of PDTC. Such agents may provide a novel approach for control of adhesion molecule gene expression in inflammation.

Leukocyte adhesion to vascular endothelium, an important step for leukocyte recruitment in inflammation and atherogenesis, is mediated by expression of specific adhesion molecules on the surface of vascular endothelial cells (1-4). Three major adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin can be induced on endothelial cells by inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1(5). TNF mediated transactivation of adhesion molecule genes involves activation of NFkB related transcriptional factors that recognize the kB regulatory sequence found in several genes including VCAM-1, ICAM-1 and E-Selectin (6-8).

5-Methoxy-3-(1-methylethoxy)benzo[b]thiophene-2-carboxamide-1-oxide (PD 144795) has previously been shown inhibit leukocyte adhesion to endothelium (9,10). The molecular mechanism of action of this compound is not well understood. We examined the effect of PD 144795 on TNF-induced expression of VCAM-1 and ICAM-1 in human aortic endothelial cells (HAEC). m-RNA and protein levels of both adhesion molecules were decreased by the

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compound. Furthermore, unlike the antioxidant PDTC (11), the inhibitory effect of PD 144795 was not mediated by blocking NFkB activation, suggesting a novel mechanism of action distinct from PDTC.

Materials and Methods

Cell culture: HAEC (Cell System, Kirkland, WA) were cultured in a 50:50 mixture of CS 3.0 media (Cell System) and MCDB-107 (Sigma Chemical Co. St.Louis, MO) containing 10% fetal calf serum.

Measurement of cell-surface ICAM-1 and VCAM-1: HAEC were treated with TNF-α (250 U/ml; Genzyme, Cambridge, MA) overnight at 37°C in the presence or absence of various compounds dissolved in DMSO (0.005% final concentration). Cells were washed with 2%bovine serum albumin (BSA) containing Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) and incubated with anti-VCAM-1 (1.25 µg/ml,BBA6, R and D system, Minneapolis,MN) or anti-ICAM-1 antibodies (0.5 µg/ml,BBA3, R and D system) for 2 h at 37°C. The unbound antibody was aspirated and cells washed three times with 2%BSA-DMEM. For detection of VCAM-1 the cells were incubated with biotin conjugated goat anti-mouse IgG, Fab fragments (#115-066-068, Jackson Immuno Research Lab, West Grove, PA) at a 1:1000 dilution for 1 h at 37°C. [^{125}I]-streptavidin (1:60 dilution,#IM.236, Amersham, Arlington Heights, IL) was then added and cells incubated for 15 min. Cells were washed and digested overnight with 1N NaOH and radioactivity in the digests counted. Cell-surface VCAM-1 expression is shown as radioactivity bound to cells. For detection of ICAM-1, the cells were incubated with a sheep anti-mouse antibody coupled to horseradish peroxidase (1:3000 dilution, #55558, Cappel, West Chester,PA) for 1h at 37°C and then color reagent added. The absorbance was read at 414 nm.

Northern blot analysis: Total RNA from HAEC treated with TNF in the presence or absence of agents was isolated using guanidium thiocyanate-phenol-chloroform (12). RNA was separated on a 1% agarose formaldehyde denaturing gel in the presence of ethidium bromide and then transferred to a nitrocellulose membrane by capillary transfer. The membrane was pre-hybridized at 42°C for 1h followed by hybridization in 5X SSPE (1X= 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 50% Formamide, 5X Denhardt's, 0.2% SDS, 200 µg/ml salmon testes DNA and 0.2 vol 50% dextran sulfate. [^{32}P]-labeled probes were prepared using the random primer oligonucleotide method (13). Approximately 3-5 X 10^6 CPM of labeled VCAM-1 or ICAM-1 or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probes were used per hybridization. Membranes were washed with a final stringency of 0.2X SSC (1X= 150 mM NaCl, 15 mM Na Citrate) 0.1% SDS at 55°C and subjected to autoradiography at -70°C.

Electrophoretic mobility shift assay: HAEC were pretreated with PD 144795 or PDTC for 1 h and then exposed to TNF for an additional hour and nuclear extracts prepared (14). Two complimentary oligonucleotides containing the two NFκB sites (underlined) of the human VCAM-1 promoter (49-mers, Oligos Etc. Inc. wildtype sense sequence: 5'-G CTG CCC TGG GTT TCC CCT TGA AGG GAT TTC CCT CCG CCT CTG CAA CAA-3') were annealed in 50 mM Tris pH 8.0, 100 mM NaCl and 10 mM MgCl. Double-stranded oligonucleotides were labeled on their 5' ends with [^{32}P] using T4 polynucleotide kinase (Promega) as described by the manufacturer. The DNA binding reaction was performed at 30°C for 15 min in a total reaction volume of 20-25 µl.

Results and Discussion

PD 144795 treatment of HAEC decreased the TNF-induced cell-expression of both VCAM-1 and ICAM-1 in a concentration-dependent manner (figure 1) with IC_{50} values of 4 µM and

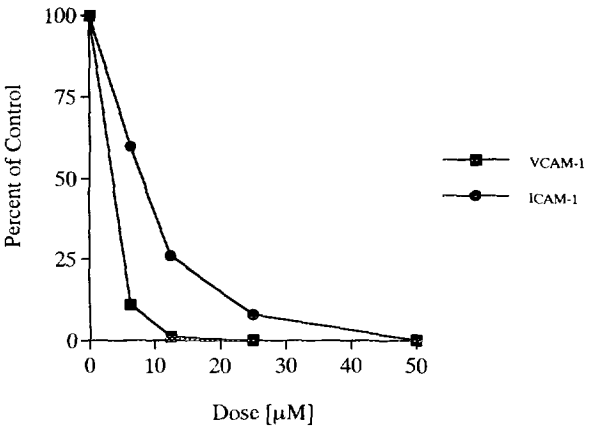

Figure 1. Effect of PD 144795 on adhesion molecule expression. HAEC were incubated overnight with TNF (250 U/ml) together with increasing concentrations of PD 144795 and cell-surface expression of VCAM-1 or ICAM-1 determined using immunoassays. Results are shown as percent of control (100% control=21 and 8-fold increase in cell-surface expression of VCAM-1 and ICAM-1 respectively, by TNF treatment relative to unstimulated cells). Experiments were performed in triplicate wells and data shown are average.

10 μM respectively. Protein synthesis measured using tritiated leucine incorporation in HAEC treated with PD 144795 for 12h in the presence of TNF was not affected up to the maximum concentration tested (50 μM; data not shown) suggesting that the compound was not cytotoxic.

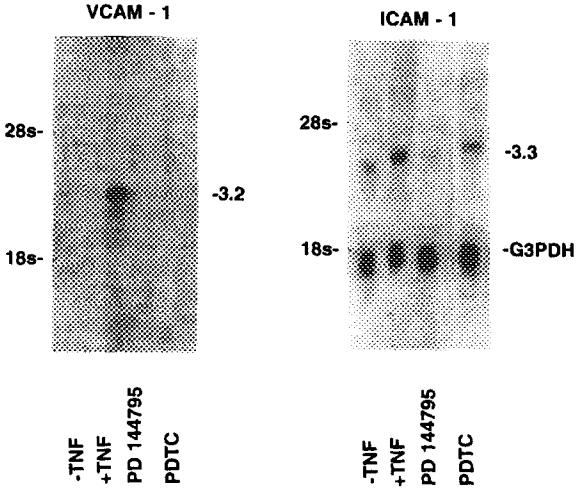

Figure 2. Effects of PD 144795 and PDTC on adhesion molecule mRNA. Total RNA (25 μg) from HAEC treated for 6 h with media alone (- TNF) or TNF, 250 U/ml (+TNF) or TNF and 50 μM PD144795 (PD144795) or TNF and 100 μM PDTC (PDTC) was isolated and northern blot analysis for VCAM-1 (left panel) or ICAM-1 and G3PDH (right panel) was performed.

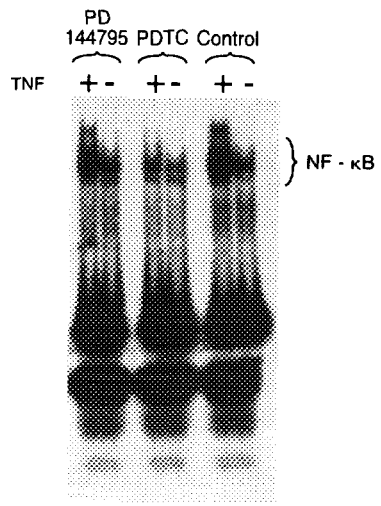

Figure 3. Effect of PD 144795 and PDTC on NFkB activation. HAEC were pretreated for 1h with either media alone or PD 144795 (50μM) or PDTC (100μM) followed by 1h incubation with or without TNF in the continued presence of the compounds. Nuclear proteins were prepared and gel-shift assays were performed as described in materials and methods.

PD 144795 inhibition of adhesion molecule expression was not related to blocking TNF interaction with its receptor since VCAM-1 and ICAM-1 cell-surface expression induced by a non-cytokine stimulus, lipopolysaccharide, was also inhibited by PD 144795 (data not shown). Northern blot analysis of total RNA from HAEC treated with TNF showed that the cytokine markedly increased the m-RNA levels of VCAM-1 and ICAM-1 and PD 144795 treatment resulted greater than 90% reduction in the mRNA levels for both adhesion molecules but did not affect the constitutively expressed G3PDH m-RNA (figure 2). These data suggest that the compound effects transcription of the two adhesion molecule genes. Treatment with PDTC, an antioxidant previously reported to decrease cell-surface expression of adhesion molecules (14), also decreased VCAM-1 m-RNA and slightly decreased ICAM-1 mRNA levels (figure 2).

Two major bands representing NFkB binding activity induced by TNF were observed using mobility shift assays (figure 3). PDTC treatment resulted in greater than 90% inhibition of this binding activity, but PD 144795 treatment had no effect, suggesting that NFkB activation is not affected by this compound.

In conclusion, PD 144795 potently inhibits cytokine-induced expression of VCAM-1 and ICAM-1 likely through an NFkB-independent pathway which in part may contribute to its anti-inflammatory effects.

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