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Phenyl Methimazole Inhibits TNF-α-Induced VCAM-1 Expression in an IFN Regulatory Factor-1-Dependent Manner and Reduces Monocytic Cell Adhesion to Endothelial Cells[1]

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Proinflammatory cytokine (e.g., TNF-α)-induced expression of endothelial cell adhesion molecules (ECAMs) on the lumenal surface of the vascular endothelium and a consequent increase in leukocyte adhesion are key aspects of pathological inflammation. A promising therapeutic approach to diminish aberrant leukocyte adhesion is, therefore, to inhibit cytokine-induced ECAM expression at the transcription level. Several studies suggest that methimazole, a compound used clinically to treat autoimmune diseases, such as Graves' disease, may also diminish pathological inflammation by suppressing ECAM expression. In this study we probed the hypothesis that a derivative of methimazole, phenyl methimazole (compound 10), can reduce cytokine-induced ECAM expression and consequent leukocyte adhesion. We found that compound 10 1) dramatically inhibits TNF-α-induced VCAM-1 mRNA and protein expression in human aortic endothelial cells (HAEC), has a relatively modest inhibitory effect on TNF-α induced E-selectin expression and has no effect on ICAM-1 expression; 2) significantly reduces TNF-α-induced monocytic (U937) cell adhesion to HAEC under in vitro flow conditions similar to that present in vivo; 3) inhibits TNF-α-induced IFN regulatory factor-1 binding to VCAM-1 promoter; and 4) reduces TNF-α-induced IRF-1 expression in HAEC. Combined, the results indicate that phenyl methimazole can reduce TNF-α-induced VCAM-1 expression in an IFN regulatory factor-1-dependent manner and that this contributes significantly to reduced monocytic cell adhesion to TNF-α-activated HAEC. ***The Journal of Immunology*****, 2004, 173: 2041–2049.**

The adhesion of leukocytes to the endothelium in the fluid dynamic environment of the circulation plays a central role in pathological inflammation (e.g., atherosclerosis (1) and inflammatory bowel disease (2)). Endothelial cell adhesion molecules (ECAMs)[4] known to participate in leukocyte recruitment, (e.g., VCAM-1, E-selectin, and ICAM-1) have been shown to be up-regulated in such settings and to contribute to disease progression and/or tissue damage by virtue of their role in leukocyte adhesion (3). For example, VCAM-1 is present in a localized fashion on aortic endothelium that overlies early foam cell lesions (1) and is increased on endothelium in models of colitis (4). A promising therapeutic approach for treating pathological inflammation, therefore, is to reduce aberrant leukocyte adhesion to the endothelium via suppression of ECAM expression (5).

ECAM expression is influenced by the cytokine milieu in which the endothelial cells reside. Indeed, treating cultured endothelial cells with the proinflammatory cytokine TNF-α elicits the expression of E-selectin, VCAM-1, and ICAM-1 (6). The cytokine-dependent ECAM induction is regulated at the gene level by the activity of transcription factors such as NF-κB, AP-1, specificity protein-1 (SP-1), IFN regulatory factor-1 (IRF-1), and GATA. For example, the E-selectin promoter has binding sites for NF-κB (7); the VCAM-1 promoter has binding sites for NF-κB, AP-1, SP-1, IRF-1, and GATA (8–11); and the ICAM-1 promoter has functional binding sites for NF-κB and AP-1 (12, 13). Some of these transcription factors (e.g., NF-κB) are present in unstimulated endothelial cells in an inactive form (14); cytokine treatment stimulates their activity (14) and induces the expression of other transcription factors (e.g., IRF-1) (10). The active/induced *trans* factors bind to their respective *cis* elements, leading to gene transcription.

Current and potential therapies for pathological inflammation work at least in part by modulating the activity of transcription factors (15–19). Indeed, compounds that block cytokine-induced ECAM expression at the transcription level have been shown to inhibit leukocyte adhesion to the endothelium (16–18, 20) and to reduce inflammation in animal models (15, 17). Methimazole is widely used clinically for the treatment of autoimmune Graves' disease or primary hyperthyroidism (21) and has been shown to be effective in treating several other forms of autoimmune diseases: psoriasis in humans (22), systemic lupus, autoimmune blepharitis, autoimmune uveitis, thyroiditis, and diabetes in murine experimental models (23–26). Evidence has accumulated that methimazole acts as a transcriptional inhibitor of abnormally increased MHC class I and class II gene expression (26–29) and mimics the effect of a class I knockout in preventing autoimmune disease (30).

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[4] Abbreviations used in this paper: ECAM, endothelial cell adhesion molecule; C10, phenyl methimazole (compound 10); HAEC, human aortic endothelial cells; HBSS$^+$, HBSS with Ca^{2+} and Mg^{2+}; IRF-1, IFN regulatory factor-1; M199, medium 199; SP-1, specificity protein-1.

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Several observations suggest that methimazole may also affect ECAM expression and thus could be a potential anti-inflammatory compound. Specifically, it has been reported that 1) Graves' disease patients treated with methimazole have reduced levels of circulating soluble E-selectin and soluble VCAM-1 (31); and 2) methimazole decreases colonic mucosal damage in a rat model of experimental colitis (32). An effort to identify derivative compounds with greater efficacy than methimazole led to the observation that phenyl methimazole (compound 10 (C10)), a tautomeric cyclic thione, was 50- to 100-fold more potent at suppressing abnormally increased MHC gene expression and was a far more effective agent in experimental models of lupus and diabetes (26, 28). These observations motivated us to probe the hypothesis that phenyl methimazole can reduce proinflammatory (e.g., TNF-α)-induced ECAM expression and consequent leukocyte adhesion to endothelial cells.

Materials and Methods

Materials

Medium 199 (M199), RPMI 1640 (RPMI), and HBSS with Ca^{2+} and Mg^{2+} ($HBSS^{+}$) were obtained from BioWhittaker (Walkersville, MD). Heat-inactivated FBS was purchased from HyClone (Logan, UT); endothelial growth factor and recombinant human TNF-α were obtained from Calbiochem (San Diego, CA); bovine hypothalamus extract was purchased from Pel-Freeze Biologicals (Rogers, AR); Dulbecco's PBS without Ca^{2+} or Mg^{2+} (PBS), gelatin, heparin, DMSO, BSA, *O*-phenylenediamine dihydrochloride, phosphate citrate buffer tablets with sodium perborate, L-glutamine, trypsin-versene, penicillin/streptomycin, and nonessential amino acids were obtained from Sigma-Aldrich (St. Louis, MO) or were the highest purity available. The assay buffer was $HBSS^{+}$/0.5% BSA. C10 was synthesized as described by Ricerca (Cleveland, OH) (26) and prepared as a 200-mM stock solution in DMSO.

Antibodies

Function-blocking murine mAb HEL3/2 (anti-E-selectin; IgG1) was a gift from Dr. R. T. Camphausen (Wyeth Laboratories, Cambridge, MA). Function-blocking murine mAb 51-10C9 (anti-VCAM-1; IgG1) was obtained from BD Pharmingen (San Diego, CA). Murine mAb R6.5 (anti-ICAM-1; IgG2a) was provided by Dr. R. Rothlein (Boehringer Ingelheim, Ridgefield, CT). Murine mAb TS1/22 (anti-LFA-1; IgG1) was obtained from Endogen (Woburn, MA). HRP-conjugated goat $F(ab')_2$ anti-mouse IgG polyclonal secondary Ab (Calbiochem) was used to detect the primary mAbs in the ELISA. The polyclonal Abs to p50 (sc-1191), p65 (sc-109), p52 (sc-298), c-Rel (sc-70), RelB (sc-226), and IRF-1 (sc-1041X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatment of HAEC

Human aortic endothelial cells (HAEC; Cambrex Bio Science Walkersville, Walkersville, MD) were cultured in M199 supplemented with 8% FBS, 100 μg/ml heparin, 10 ng/ml endothelial growth factor, 100 μg/ml hypothalamus extract, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. HAEC were subcultured on gelatin-precoated, 96-well culture plates (Corning Glass, Corning, NY) for viability assays and ELISA; 100-mm culture dishes (BD Falcon, Franklin Lakes, NJ) for Northern blot analyses, Western blot analyses and EMSA; 35-mm culture dishes (Corning Glass) for adhesion assays; and 24-well culture plates for luciferase promoter assays (BD Falcon). All experiments were performed with confluent HAEC monolayers. Unless noted otherwise, HAEC were treated with 25 ng/ml TNF-α in the absence or the presence of C10 or 0.25% DMSO (carrier control for C10), for 2–24 h; the concentration of DMSO was held constant at 0.25% (unless indicated otherwise). Treatment of HAEC with C10 at the concentrations used in this study had little or no effect on HAEC viability as determined by MTS assay (20) and visual inspection of the HAEC monolayers (data not shown).

U937 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI supplemented with 8% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the adhesion assays, U937 cells were washed, resuspended to 1×10^8 cells/ml in RPMI, and held on ice (<4 h) until used in the adhesion assay.

ELISA

The ELISA was similar to that described previously (20). HAEC were washed, fixed in 1% formaldehyde at 4°C for 20 min, washed, and incubated in M199 containing 8% FBS. All Ab dilutions and washes were with M199/8% FBS. Murine mAbs were added (10 μg/ml), and the HAEC were incubated at 4°C for 20 min. After the incubation, the wells were washed, and a peroxidase-conjugated polyclonal Ab to mouse IgG was added (diluted 1/50). After a 20-min incubation at 4°C, the wells were washed and treated with *O*-phenylenediamine dihydrochloride dissolved in phosphate citrate buffer containing sodium perborate. After a 10-min incubation, the absorbance of each well was determined at 450 nm using a microwell plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

RNA isolation and Northern blot analysis

Northern blot analysis was performed in a manner similar to that described previously (27, 29, 33). HAEC were washed with PBS, and total RNA was extracted using a commercial kit (RNeasy Mini Kit; Qiagen, Valencia, CA). Twelve micrograms of total RNA per lane was resolved on 1% denaturing agarose gels containing 0.66 M formaldehyde. Gels were capillary-blotted on Nytran membranes (Schleicher & Schuell, Keene, NH), UV-cross-linked, and used for hybridization. The probe for IRF-1 has been described previously (33); G3PDH cDNA was obtained from BD Clontech (Palo Alto, CA). Other probes were synthesized by RT-PCR (33) using the following specific primers: VCAM-1, 5′-GACTCCGTCTCATTGACTTGCAGCACCACAG-3′ and 5′-ATACTCCCGCATCCTTCAACTGGGCCTTTCG-3′ (1876 bp); E-selectin, 5′-GTGCAGCCATTCCCCTGCTGGAGAGTTC-3′ and 5′GGGCCAGAGACCCGAGGAGAGTTATCTG-3′ (977 bp); and ICAM-1, 5′-CTCAGGTATCCATCCATCCCAGAGAAGCCTTCC-3′ and 5′-CCCTTGAGTTTTATGGCCTCCTCCTGAGCCTTC-3′ (1514 bp). The cDNAs were labeled with [α-^{32}P]dCTP using the Ladderman Labeling Kit (Takara Biochemical, Berkeley, CA) (33). Northern blots were developed using a BAS 1500 Bioimaging Analyzer (Fuji Photo Film Medical Systems USA, Stamford, CT). Each experiment was repeated at least twice.

Nuclear extracts and EMSA

Nuclear extracts were prepared from harvested HAEC using NE-PER® extraction reagents (Pierce, Rockford, IL) in the presence of a protease inhibitor mixture (PMSF, leupeptin, and pepstatin A). Oligonucleotides (Biosynthesis, Lewisville, TX) were annealed, and precipitated double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Binding reactions (20 min, room temperature) included ^{32}P-labeled probe (activity, 100,000 cpm), 3–6 μg of HAEC nuclear extract, 1 μg of poly(dI-dC), 1 mM DTT, 10% glycerol, and 1× binding buffer. The binding buffer (10×) used for the NF-κB EMSA was 200 mM HEPES-KOH (pH 7.9), 340 mM KCl, 50 mM $MgCl_2$, 5 mM EDTA (pH 8.0), and 1% Triton X-100. The binding buffer (10×) used for the IRF-1 EMSA was 100 mM Tris-HCl (pH 7.5), 500 mM NaCl, 50 mM $MgCl_2$, and 10 mM EDTA (pH 8.0). In competition studies, nuclear extracts were incubated with a 100-fold molar excess of unlabeled double-stranded oligonucleotide. In Ab studies, nuclear extracts were incubated with 2 μg of the appropriate Abs. After the incubations, reaction mixtures were electrophoresed (160 V, room temperature) on 5% nondenaturing polyacrylamide gels containing 5% glycerol in 1× TBE (50 mM Tris, 50 mM boric acid, and 1 mM EDTA). Gels were dried and autoradiographed. Each experiment was replicated at least twice.

Flow adhesion assays

A parallel plate flow chamber (Glycotech, Rockville, MD) was used and has been previously described (20). U937 cells (5×10^5 cells/ml in assay buffer) were drawn over the HAEC at a shear of 1.8 dynes/cm^2. To determine U937 cell accumulation, the number of U937 cells adherent (either rolling or firmly adherent) to the HAEC monolayer in eight different fields of view after 2.5 min of flow was determined, averaged, and normalized to the area of the field of view to give the result for that particular run. Such an assay was performed at least three times, and the values were averaged to give the results presented in the figures. In certain experiments, TNF-α-activated HAEC were pretreated (37°C for 15 min before assay) with 10 μg/ml mAbs; this concentration of mAbs was used because preliminary experiments indicated that it was maximally effective.

Dual luciferase assay

Four human VCAM-1 promoter deletion constructs of different lengths (−1641/+12, −288/+12, −228/+12, and −85/+12 bp) were amplified by PCR from human genomic DNA. Each upstream primer contained a restriction endonuclease *Mlu*I site located at the 5′ end of the primer. The downstream primer corresponding to the +12 end contained a restriction

endonuclease *Xho*I site at the 5′ end. The PCR products were digested by *Mlu*I and *Xho*I (New England Biolabs, Beverly, MA) and ligated into a similarly digested pGL3 basic luciferase reporter vector (Promega, Madison, WI). Cells were transfected for 24 h with 400 ng of the indicated constructs or pGL3 basic luciferase reporter vector as control, using GeneJuice transfection reagent (Novagen, Madison, WI). All cells were also transfected with phRL-TK (Int-) vector (Promega), which contains wild-type *Renilla* luciferase (R*luc*), as an internal transfection control. Luciferase assays were conducted with the Dual-Luciferase Reporter Assay System (Promega) on a Berthold Lumat LB 9507 tube luminometer (PerkinElmer, Rockville, MD).

Western blot analysis

Whole cell lysates were prepared in lysis buffer (150 mM NaCl, 1% IGEPAL CA-630, and 50 mM Tris-HCl, pH 8.0). Lysates (20 μg) were then resolved on 4–12% bis-Tris PAGE gels under denaturing conditions using the NuPAGE Bis-Tris System (Invitrogen Life Technologies, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes, which were probed with IRF-1 Ab. Subsequent binding of HRP-conjugated goat anti-rabbit Ab (sc-2054; Santa Cruz Biotechnology) was detected using Lumigen PS-3 detection reagents (Amersham Biosciences, Piscataway, NJ).

Statistics

A single-factor ANOVA was used to assess statistical differences. If ANOVA indicated significant differences between conditions, Bonferroni's test was used for multiple pairwise comparisons. Student's *t* test was used to assess differences in luciferase promoter assays. Values of $p < 0.001$ (for ELISA) and $p < 0.05$ (for adhesion and luciferase promoter assays) were considered significant. Unless stated otherwise, all error bars represent the SD.

Results

C10 dramatically inhibits TNF-α-induced VCAM-1 expression, has a modest effect on E-selectin expression, and has no effect on ICAM-1 expression

We sought to determine the effect of C10 on TNF-α induced ECAM expression using HAEC. Because the ECAM profile on 4- and 24-h TNF-α-treated endothelial cells can be significantly different (6), we investigated the effect of C10 on 4- and 24-h TNF-α-treated HAEC. As shown in Fig. 1*A*, unactivated HAEC did not appear to express E-selectin or VCAM-1, but did express ICAM-1; 4-h treatment of HAEC with TNF-α induced E-selectin and VCAM-1 protein expression and significantly increased ICAM-1 protein expression (Fig. 1*A*). Treatment of HAEC with DMSO (carrier control) had little or no effect on 4-h TNF-α-induced protein expression of E-selectin, ICAM-1, and VCAM-1 (Fig. 1*A*). Treatment of HAEC with C10 at concentrations $\geq$0.25 mM significantly reduced 4-h TNF-α induced protein expression of VCAM-1, but had little, if any, effect on 4-h TNF-α-induced protein expression of E-selectin and ICAM-1 (Fig. 1*A*). The expression at the protein level was paralleled at the mRNA level. Specifically, Northern blot analyses revealed that C10 reduced, in a dose-dependent manner, TNF-α-induced VCAM-1 mRNA expression, had a marginal effect on E-selectin mRNA expression, and had no effect on ICAM-1 mRNA expression (Fig. 1*B*).

The ability of C10 to inhibit TNF-α-induced VCAM-1 protein and mRNA was also observed at 24 h. In addition, an inhibitory effect on E-selectin expression was observed. As shown in Fig. 2*A*, HAEC treated with TNF-α for 24 h expressed a level of E-selectin that was higher than the basal level (Fig. 2*A*), although distinctly less than the level seen at 4 h post-NF-α treatment (Fig. 1*A*). Twenty-four-hour TNF-α-activated HAEC also expressed elevated levels of ICAM-1 and VCAM-1 (relative to unactivated HAEC; Fig. 2*A*). Treatment of HAEC with DMSO had little or no effect on the 24-h TNF-α-induced expression of E-selectin, ICAM-1, and VCAM-1 (Fig. 2*A*). In contrast, treatment of HAEC with C10 at concentrations $\geq$0.05 mM significantly reduced the 24-h TNF-α-induced expression of E-selectin and VCAM-1, but had no effect on ICAM-1 expression (Fig. 2*A*). Again, expression

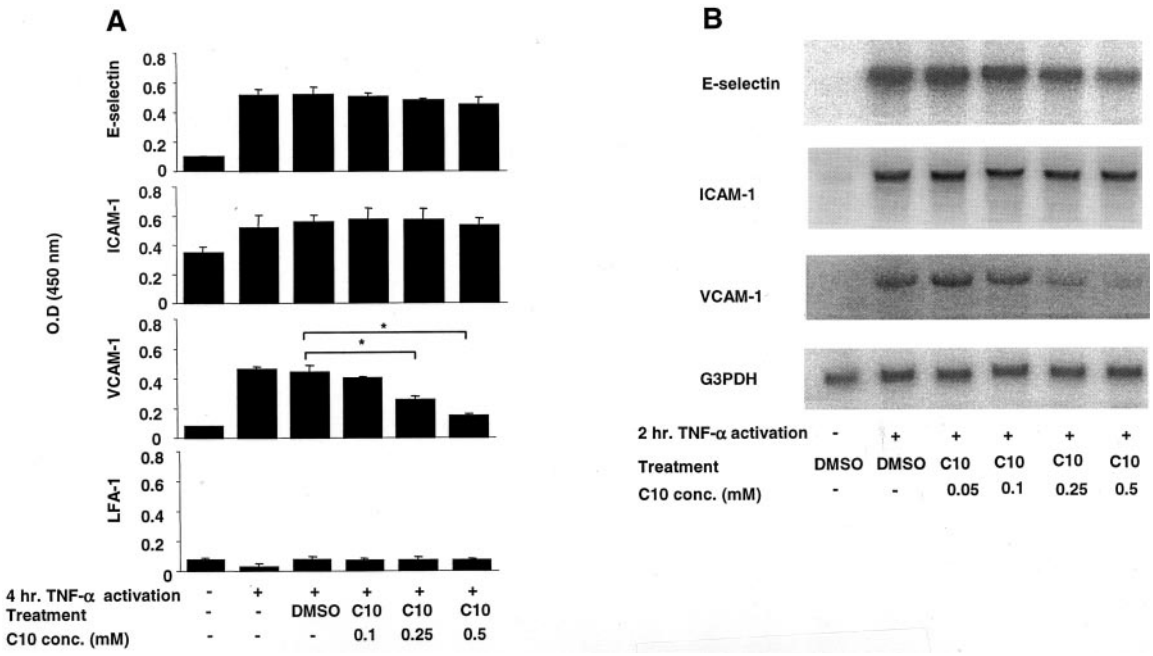

FIGURE 1. C10 significantly inhibits short term (2–4 h) TNF-α-induced expression of VCAM-1, but has little or no effect on TNF-α-induced E-selectin and ICAM-1 expression. The protein (*A*) and mRNA (*B*) levels of E-selectin, ICAM-1, and VCAM-1 on unactivated and 4-h (ELISA) or 2-h (mRNA) TNF-α-activated HAEC in the absence or the presence of C10 were determined by ELISA and Northern blot analysis, respectively. *A*, The level of absorbance indicated by OD (at 450 nm) correlates with the level of a given ECAM protein (e.g., E-selectin) on the HAEC. All values are the mean $\pm$ SD of triplicate wells. The results presented are representative of a typical experiment performed at least three times. A mAb to LFA-1 (TS1/22) served as a negative control. *B*, RNA isolated from HAEC was subjected to Northern blot analyses using appropriate probes for E-selectin, ICAM-1, and VCAM-1. The G3PDH probe was used as the loading control. The results presented are typical of three separate experiments. $*$, $p < 0.001$.

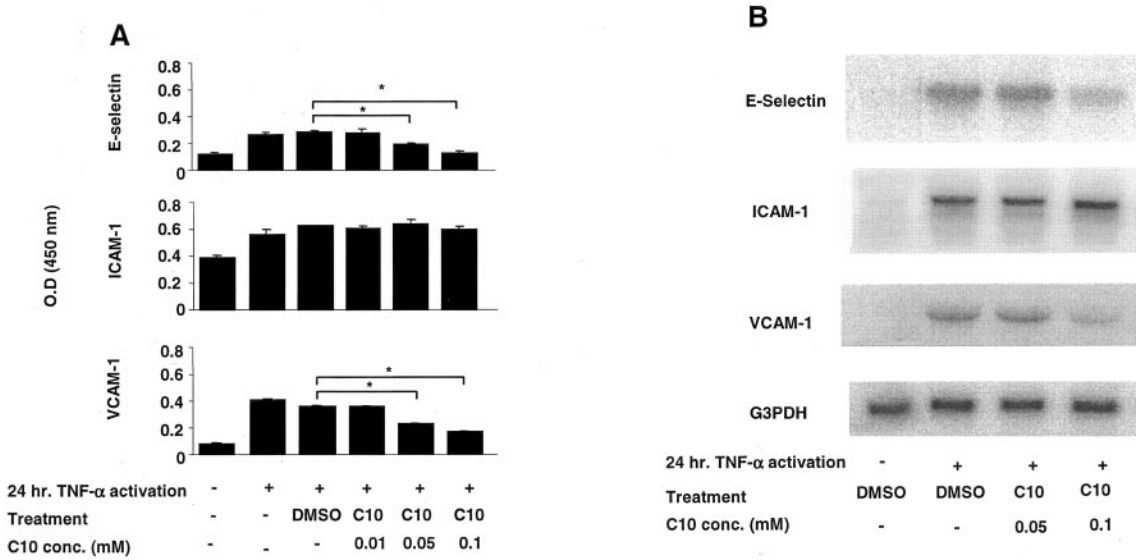

FIGURE 2. C10 significantly inhibits 24-h TNF-α-induced expression of VCAM-1 and E-selectin, but has no effect on ICAM-1 expression. The protein (*A*) and mRNA (*B*) levels of E-selectin, ICAM-1, and VCAM-1 on unactivated and 24-h TNF-α-activated HAEC in the absence or the presence of C10 were determined by ELISA and Northern blot analysis, respectively, as in Fig. 1. *, $p < 0.001$.

at the protein level was paralleled at the mRNA level. Specifically, Northern blot analyses revealed that C10 reduced, in a dose-dependent manner, 24-h TNF-α-induced E-selectin and VCAM-1 mRNA, whereas it had little or no effect on ICAM-1 mRNA expression (Fig. 2*B*).

C10 inhibits monocytic cell adhesion to TNF-α-activated HAEC

We probed the effect of C10 on monocytic (U937) cell adhesion to 4- and 24-h TNF-α-activated HAEC. We used an in vitro flow chamber that mimics flow conditions present in vivo, a mAb-blocking approach to determine which ECAMs were involved in the adhesion of U937 cells to TNF-α activated HAEC, and C10 concentrations (0.5 mM for 4 h and 0.1 mM for 24 h) that had maximal effects in our ELISAs (Figs. 1*A* and 2*A*). A significant number of U937 cells adhered to 4-h TNF-α-activated HAEC (Fig. 3*A*, *column 2*), whereas very few, if any, U937 cells adhered to unactivated HAEC (Fig. 3*A*, *column 1*). U937 cell adhesion was unaffected by treatment of 4-h TNF-α-activated HAEC with 51-10C9, a function-blocking mAb to VCAM-1 (*column 3* vs *column 2*; Fig. 3*A*) and was partially reduced by treatment of 4-h TNF-α-activated HAEC with HEL 3/2, a function-blocking mAb to E-selectin (Fig. 3*A*, *column 4* vs *column 2*). A further reduction in U937 cell adhesion was seen upon treatment of 4-h TNF-α-activated HAEC with a combination of 51-10C9 and HEL3/2 (Fig. 3*A*, *column 5* vs *column 4*). Combined, these results suggest that U937 cell adhesion to 4-h TNF-α-activated HAEC is mediated by both E-selectin and VCAM-1. This is consistent with other reports (18).

The combination of C10 and the mAb to E-selectin (HEL3/2) significantly reduced 4-h TNF-α-induced U937 cell adhesion relative to treatment with the mAb to E-selectin alone (Fig. 3*A*, *column 6* vs *column 4*). Treatment of HAEC with C10 alone had little effect on the 4-h TNF-α-induced U937 cell adhesion compared with treatment with DMSO (Fig. 3*A*, *column 7* vs *column 8*). Combined, these data demonstrate that C10 has a modest effect on 4-h TNF-α-induced U937 cell adhesion to HAEC, consistent with the mAb data presented above showing that inhibition required mAbs to E-selectin and VCAM-1 and with the evidence presented in Fig. 1 showing that at 4 h, C10 had a selective effect on VCAM-1 mRNA and protein expression.

A more dramatic effect was observed at the 24 h TNF-α activation point (Fig. 3*B*). A significant number of U937 cells adhered to 24-h TNF-α-activated HAEC (Fig. 3*B*, *column 2*), whereas very

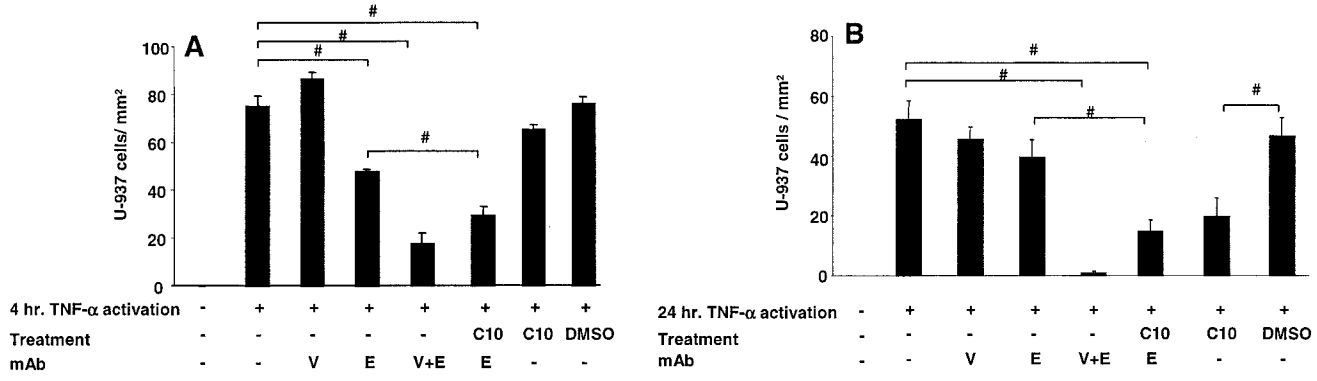

FIGURE 3. C10 has a modest effect on U937 adhesion to 4-h TNF-α-activated HAEC and a dramatic effect on U937 adhesion to 24-h TNF-α-activated HAEC. HAEC were treated for 4 h (*A*) or 24 h (*B*) with TNF-α in the absence or the presence of C10. In certain instances, HAEC were pretreated with mAb before use in adhesion assays. mAb indicates pretreatment of HAEC with a mAb to E-selectin (E), VCAM-1 (V), or a combination (V+E) after the other treatments, but before the adhesion assay. $n \geq 3$. error bars are the SEM. #, $p < 0.05$.

few, if any, U937 cells adhered to unactivated HAEC (Fig. 3*B*, *column 1*). The U937 cell adhesion to 24-h TNF-α-activated HAEC was dependent on both E-selectin and VCAM-1 (Fig. 3*B*, *column 5* vs *columns 2–4*). The combination of C10 and the mAb to E-selectin (HEL3/2) significantly reduced 24-h TNF-α-induced U937 cell adhesion (Fig. 3*B*, *column 6* vs *column 2*). In addition, the combination of C10 and the mAb to E-selectin significantly reduced the 24-h TNF-α-induced U937 cell adhesion relative to treatment with the mAb to E-selectin alone (Fig. 3*B*, *column 6* vs *column 4*). Treatment of HAEC with C10 alone also significantly reduced the 24-h TNF-α-induced U937 cell adhesion relative to treatment with DMSO (Fig. 3*B*, *column 7* vs *column 8*). Combined, the above results clearly demonstrate that C10 significantly reduces long term (24-h) TNF-α-induced U937 cell adhesion to HAEC.

C10 affects VCAM-1 gene transcription

Because 1) the effect of C10 appeared to be more dramatic on VCAM-1 expression than on E-selectin expression; 2) our adhesion data (Fig. 3) and those from other laboratories (18) have implicated VCAM-1 in monocytic cell adhesion to TNF-α-activated endothelial cells; and 3) VCAM-1 has been implicated in important pathologies (e.g., atherosclerosis) (1), we chose to focus on the effect of C10 on VCAM-1 expression.

To probe whether C10 acts transcriptionally to inhibit TNF-α-induced VCAM-1 gene expression and also to get an idea of the molecular mechanism of C10's inhibitory action, we conducted VCAM-1 promoter reporter assays. The locations of the *cis* elements known to play a role in TNF-α-induced human VCAM-1 expression (NF-κB, AP-1, SP-1, IRF-1, and GATA) lie between −1641 and +12, as noted in Fig. 4 (8–11). Four truncations of the VCAM-1 transcriptional regulatory element were created (−1641/+12, −288/+12, −228/+12, and −85/+12 bp constructs) in an attempt to grossly separate their activities (Fig. 4). These were then inserted into a luciferase reporter plasmid and transfected into HAEC.

TNF-α treatment induced an increase in promoter activity of each of the four constructs (Fig. 4). C10 treatment inhibited the TNF-α-induced activity of all four constructs (Fig. 4) in the absence of a consistent significant effect on basal promoter activity (Fig. 4). Note that although the TNF-α-induced increase in promoter activity decreased between the −228/+12 and −85/+12 bp constructs (Fig. 4), an inhibitory effect of C10 was observed with the 85/+12 bp construct (Fig. 4). Combined, these data clearly demonstrate that C10 affects VCAM-1 gene transcription and suggest that C10 acts on a transcriptional regulatory event that occurs within −85/+12 bp of the VCAM-1 promoter.

C10 does not affect TNF-α-induced NF-κB binding activity to VCAM-1 promoter

The binding sites for NF-κB in the VCAM-1 promoter are located within −85/+12 bp (Fig. 4) (8, 9, 11). To determine whether the inhibitory effect of C10 on TNF-α-induced VCAM-1 expression was a consequence of C10 inhibition of TNF-α-induced NF-κB activity, we conducted EMSA. EMSAs were performed with ^{32}P-labeled NF-κB probe and 6 μg of nuclear extract prepared from HAEC treated with or without TNF-α, in the absence or the presence of C10 (Fig. 5). After 2-h TNF-α treatment, a complex was induced (Fig. 5, *lane 5*), which was prominent by comparison with

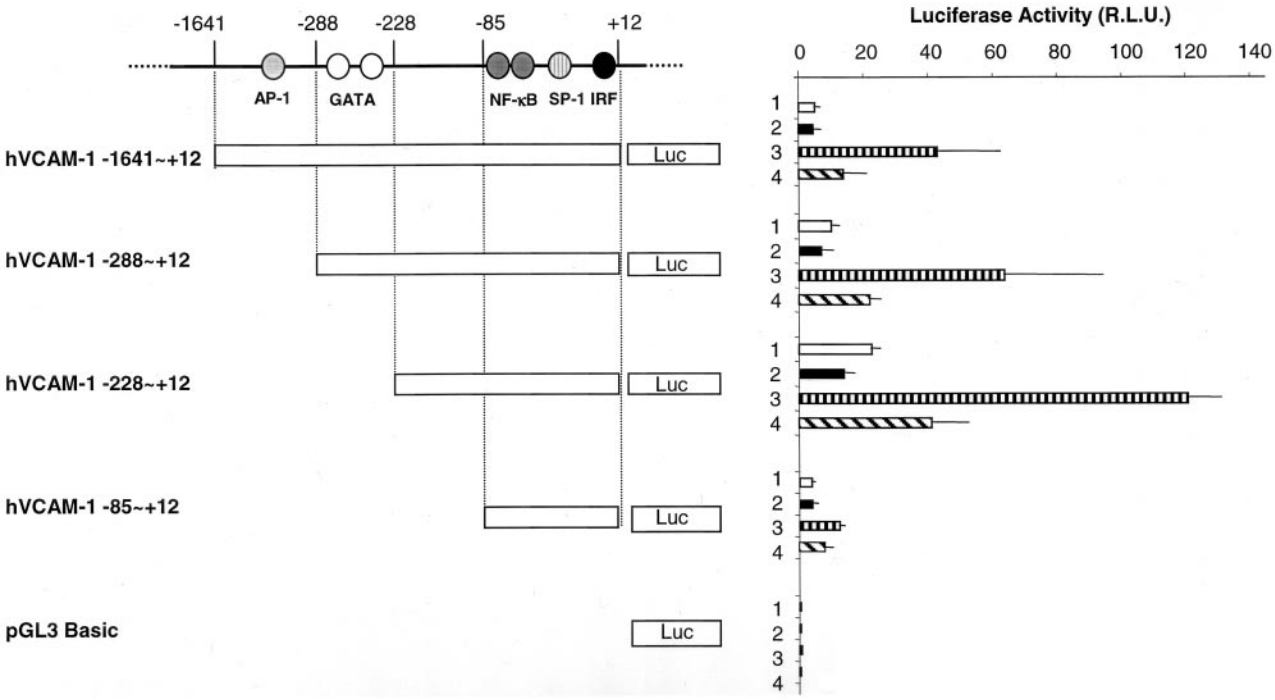

FIGURE 4. C10 inhibits the TNF-α-induced increase in VCAM-1 promoter activity in HAEC. *Left*, The locations of the binding sites for various transcription factors known to play a role in TNF-α-induced human VCAM-1 expression were used as a template to create −1641/+12, −288/+12, −228/+12, and −85/+12 bp constructs. *Right*, HAEC were transfected for 24 h with 400 ng of the constructs indicated on the *left* or pGL3 basic luciferase reporter vector. All HAEC were also transfected with phRL-TK (Int-) vector, which contains *Renilla* luciferase (R*luc*), as an internal transfection control. HAEC were treated for 6 h with 10 ng/ml TNF-α in the absence or the presence of 0.3 mM C10. All treatment conditions contained 0.3% DMSO. Assays were conducted with the Dual-Luciferase Reporter Assay System. The luciferase activity indicated by relative light units (R.L.U) correlates with the level of promoter activity. All values are the mean ± SD of triplicate wells. Results presented are typical of three separate experiments. *Bar 1*, Untreated; *bar 2*, 0.3 mM C10; *bar 3*, 10 ng/ml TNF-α; *bar 4*, 10 ng/ml TNF-α in the presence of 0.3 mM C10.

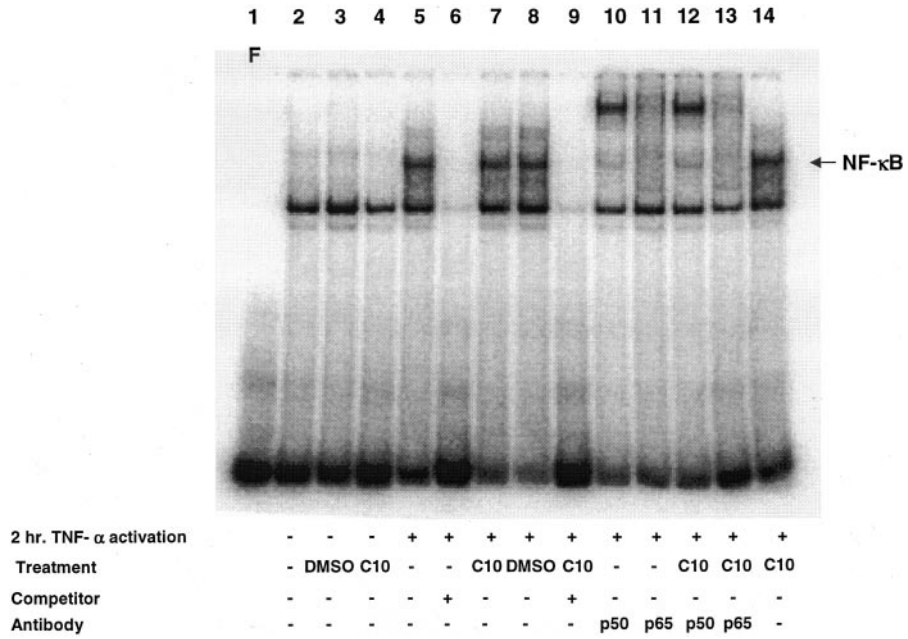

FIGURE 5. C10 does not affect 2-h TNF-α-induced NF-κB binding activity to VCAM-1 promoter. EMSAs were performed with ^{32}P-labeled NF-κB probe (hVCAM-1 NFκB: sense, 5′-TGCCCTGGGTTTCCCCTTGAA GGGATTTCCCTCCGCC-3′) and 6 μg of nuclear extracts prepared from HAEC-treated with or without TNF-α in the absence or the presence of C10. Results presented are typical of two separate experiments. Competitor indicates the presence (+) or the absence (−) of a 100-fold molar excess of unlabeled NF-κB probe.

the control, no TNF-α treatment (Fig. 5, *lane 5* vs *lane 2*). Competition with a 100-fold molar excess of unlabeled NF-κB probe eliminated the TNF-α-induced complex formation (Fig. 5, *lane 6*). Addition of 0.5 mM C10 or DMSO had no effect on TNF-α-dependent complex formation (Fig. 5, *lanes 7* and *8* vs *lane 5*). To identify the components of the complex, we used Abs to the various NF-κB subunits. An Ab directed against the p50 subunit of NF-κB inhibited formation of the TNF-α-induced complex and caused a marked supershift; an Ab directed against the p65 subunit of NF-κB also inhibited formation of the TNF-α-induced complex, but caused only a slight, compared with anti-p50, supershift (Fig. 5, *lanes 10* and *11*). The actions of both Abs on the TNF-α-induced NF-κB complex suggest that TNF-α induced the formation of a p50/p65 heterodimer complex, consistent with the findings of previous studies (10, 14). Abs directed against p52, c-Rel, and Rel-B did not inhibit complex formation (data not shown). Addition of 0.5 mM C10 also had no effect on the Ab studies (Fig. 5, *lanes 12* and *13* vs *lanes 10* and *11*). Note that DMSO or C10 treatment alone also had no effect on unactivated HAEC (Fig. 5, *lanes 3* and *4* vs *lane 2*). These data strongly suggest that the mechanism of C10 inhibition of ECAM expression is not via inhibition of NF-κB activation and binding to the VCAM-1 promoter.

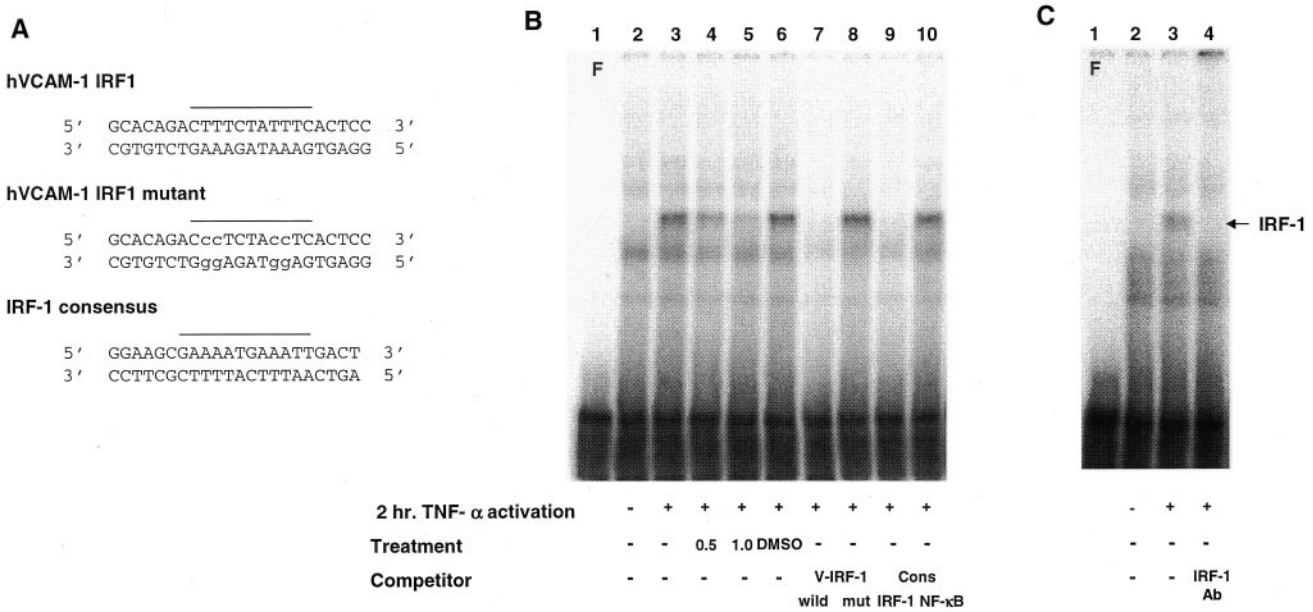

FIGURE 6. C10 inhibits 2-h TNF-α-induced IRF-1 binding activity to VCAM-1 promoter. *A*, Probe sequences used in EMSA. The overhead line indicates the IRF-1 binding site. Lowercase letters indicate the mutated bases. The sense strand sequence of the consensus NF-κB probe (not shown) is 5′-AGTTGAGGGGACTTTCCCAGGC-3′. EMSA (*B*) or supershift EMSA (*C*) were performed with ^{32}P-labeled IRF-1 probe and 3 μg of nuclear extracts prepared from HAEC treated with or without 10 ng/ml TNF-α in the absence or the presence of C10. The results presented are typical of two separate experiments. Competitor indicates the absence (−) or the presence of 100-fold molar excess of unlabeled VCAM-1 IRF-1 probe, VCAM-1 IRF-1 mutant probe, consensus IRF-1 probe, consensus NF-κB probe, or 2 μg of Ab to IRF-1.

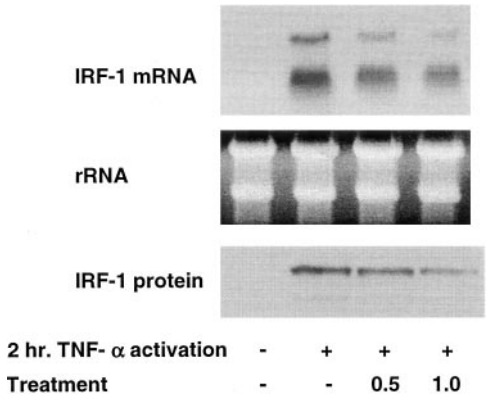

FIGURE 7. C10 reduces TNF-α-induced IRF-1 expression. The mRNA and protein levels of IRF-1 on unactivated and 2-h TNF-α-activated HAEC in the absence or the presence of C10 were determined by Northern blot and Western blot analyses, respectively, as detailed in *Materials and Methods*. Ethidium bromide-stained rRNA was the loading control for the Northern analysis. Ponceau S staining of Western blots after transfer revealed equivalent loading of total protein (data not shown).

C10 inhibits TNF-α-induced IRF-1 binding activity to VCAM-1 promoter

Because luciferase reporter assays suggested that C10 affects a transcriptional regulatory event that occurs within −85/+12 bp in the VCAM-1 promoter (Fig. 4) and because EMSA demonstrated that C10 does not affect TNF-α-induced NF-κB activation (Fig. 5), we considered the possibility that C10 might act at a different downstream site. The binding site for IRF-1 is located downstream of NF-κB binding sites within the −85/+12 bp, −11 to −1 bp, of the VCAM-1 promoter (8, 10, 11). To determine whether the inhibitory effect of C10 on TNF-α-induced VCAM-1 expression was a consequence of C10 inhibition of TNF-α-induced IRF-1 activity, we conducted EMSA. EMSAs were performed with ^{32}P-labeled IRF-1 probe (Fig. 6*A*) and 3 μg of nuclear extract prepared from HAEC treated with or without TNF-α in the absence or the presence of C10 (Fig. 6*B*). After 2-h TNF-α treatment, a complex was induced (Fig. 6*B*, *lane 3*) that was extremely prominent by comparison with the control, no TNF-α treatment (Fig. 6*B*, *lane 3* vs *lane 2*). Addition of 0.5 and 1 mM C10 inhibited the formation of TNF-α-induced complex (*lanes 4* and *5* vs *lane 3*). Note, DMSO had no effect on TNF-α dependent complex formation (Fig. 6*B*, *lane 6* vs *lane 3*). Competition with a 100-fold molar excess of unlabeled VCAM-1 IRF-1 probe (Fig. 6*B*, *lane 7*) or consensus IRF-1 probe (Fig. 6*B*, *lane 9*) eliminated TNF-α-induced complex formation. However, competition with a 100-fold molar excess of unlabeled VCAM-1 IRF-1 mutant probe (Fig. 6*B*, *lane 8*) or consensus NF-κB probe (Fig. 6*B*, *lane 10*) did not affect TNF-α-induced complex formation. Additionally (Fig. 6*C*), an Ab directed against IRF-1 inhibited formation of this TNF-α-induced complex and appeared to supershift the complex such that it barely penetrated the gel, as evidenced by a reproducibly observed dark area in top of the gel (Fig. 6*C*, *lane 4* vs *lane 3*). Combined, these data demonstrate that C10 affects IRF-1 binding activity to VCAM-1 promoter and strongly suggest that C10 inhibits VCAM-1 expression in an IRF-1-dependent manner.

C10 reduces TNF-α-induced IRF-1 expression

To further probe and determine the mechanism by which C10 inhibits TNF-α-induced IRF-1 binding to VCAM-1 promoter (Fig. 6*B*), we investigated the effects of C10 on TNF-α-induced IRF-1 protein and mRNA expression. Unactivated HAEC did not appear to express IRF-1 mRNA (Fig. 7). Two-hour treatment of HAEC with TNF-α-induced IRF-1 mRNA (Fig. 7). Addition of 0.5 or 1 mM C10 reduced the TNF-α-induced IRF-1 mRNA expression in HAEC (Fig. 7). Expression at the mRNA level was parallel to that at the protein level. Specifically, C10 treatment of HAEC reduced TNF-α-induced IRF-1 protein expression (Fig. 7). Combined, the data presented in this section demonstrate that C10 reduces TNF-α-induced IRF-1 protein and mRNA expression.

Discussion

In this study we have found that C10, a phenyl derivative of methimazole (a compound commonly used to treat autoimmune diseases, e.g., Graves' disease), has anti-inflammatory properties. Specifically, using HAEC we have found that C10 dramatically inhibits TNF-α-induced VCAM-1 mRNA and protein expression, has a relatively modest inhibitory effect on E-selectin expression, and has no effect on ICAM-1 expression. We have shown that the effect on VCAM-1 inhibition is transcriptional and have found that C10 significantly reduces TNF-α-induced monocytic cell adhesion to HAEC under in vitro flow conditions similar to those present in vivo.

The effect of the mAbs on the 4 h adhesion data (Fig. 3*A*) indicates that E-selectin and VCAM-1 are both involved in the adhesion of U937 cells to TNF-α-activated HAEC. This finding is consistent with previous reports (18) demonstrating the need for multiple mAbs to block monocytic-endothelial cell adhesion. At 4 h, C10 appears to decrease only VCAM-1 (Fig. 1); thus, it is reasonable that we see no effect on adhesion when C10 is used alone, whereas C10 is effective together with anti-E-selectin by comparison with anti-E-selectin alone (Fig. 3*A*, *column 6* vs *column 4*). We suggest anti-VCAM-1 plus anti-E-selectin is more effective than C10 plus anti-E-selectin (Fig. 3*A*, *column 5* vs *column 6*) because at this C10 concentration there is residual VCAM-1 (Fig. 1), whereas the Abs are functionally maximally effective. In contrast to the 4 h data, at 24 h, C10 reduces E-selectin as well as VCAM-1 (Fig. 2); thus, one would expect C10 to be a better inhibitor of monocytic cell adhesion than either anti-VCAM-1 or anti-E-selectin alone (Fig. 3*B*, *column 7* vs *columns 3* and *4*). However, because C10 at 24 h also does not appear to inhibit all the VCAM-1 expression (Fig. 2), a combination of anti-VCAM-1 and anti-E-selectin gives better inhibition than C10 (Fig. 3*B*, *column 5* vs *column 7*). These scenarios are reasonable interpretations of the adhesion results, particularly given the data presented in Figs. 1 and 2 and the evidence that multiple ECAMs are involved in adhesion (3, 18, 34). However, we do not exclude the possibility that more complex mechanisms, including a mechanism by which C10 reduces adhesion by affecting ECAMs in addition to VCAM-1 and E-selectin, are involved in inhibition of monocytic cell adhesion to HAEC.

Several anti-inflammatory agents diminish leukocyte adhesion by inhibiting cytokine-induced ECAM expression at the transcription level (5). Not all these compounds exert the same effect on cytokine-induced ECAM expression. For example, lactacystin can reduce the cytokine-induced expression of E-selectin, ICAM-1, and VCAM-1 (20), whereas other compounds appear to be selective for one particular ECAM (e.g., VCAM-1) (19, 35). Because the leukocyte adhesion cascade is documented to have receptor-ligand functional overlap (e.g., both E-selectin and VCAM-1 have been shown to support tethering and rolling of lymphocytes (36, 37)) compounds that suppress the expression of several of the ECAMs may be more effective at blocking leukocyte adhesion in a variety of inflammation settings. In contrast, reducing the expression of all ECAMs may cause detrimental side effects, and such a broad effect may not be necessary to achieve a therapeutic

effect. For example, VCAM-1 is present on aortic endothelium that overlies early foam cell lesions (1). Several studies suggest that antioxidants that reduce lesion development in animal models of atherosclerosis may work in part by inhibiting leukocyte adhesion (35). Indeed, it has been shown that probucol, a potent antioxidant with antiatherogenic properties, selectively reduces TNF-α-induced VCAM-1 expression and consequent monocytic cell adhesion to cultured endothelial cells (35). C10 appears to have similar effects on cultured HAEC. Specifically, C10 exerts a greater inhibitory effect on TNF-α-induced VCAM-1 expression compared with the effect on E-selectin and ICAM-1 and can diminish monocytic cell adhesion to the endothelium under flow. Further studies (i.e., animal studies) into the ability of C10 to reduce pathological inflammation, in particular atherosclerosis and colitis (pathologies that have been reported to involve VCAM-1 (1, 4)), are clearly warranted and are currently ongoing in our laboratories.

Of the VCAM-1 transcription inhibitors that have been identified, the majority of the compounds work by suppressing NF-κB activity (5, 15–18). There are however, several compounds that inhibit VCAM-1 expression via a NF-κB-independent mechanism. For example, a compound termed K-7174 selectively suppresses cytokine-induced VCAM-1 expression via a NF-κB-independent and a GATA-dependent mechanism (19). Our data suggest that C10 inhibits TNF-α-induced VCAM-1 expression in an NF-κB-independent and IRF-1-dependent manner. A previous study has shown that hyperosmotic stimuli can inhibit TNF-α-induced VCAM-1 expression in an IRF-1-dependent manner (38). It has also been reported that metal ion chelators inhibit cytokine-induced VCAM-1 expression and perhaps inhibit IRF-1 induction or activity (39). However, to our knowledge our study is the first clear demonstration of a compound that can inhibit TNF-α-induced VCAM-1 expression in an IRF-1-dependent manner and reduce monocytic cell adhesion to endothelial cells. Our observations that C10 inhibits TNF-α-induced IRF-1 expression warrant future investigation into the effect of C10 on pathological processes that involve IRF-1. Further, C10 could also be used a tool to probe the role of IRF-1 in gene regulation.

In addition to being a potential agent for the treatment of autoimmune diseases, because of its effects on abnormal MHC class I or class II gene expression, the present results suggest that the methimazole derivative, C10, has potential for the treatment of pathological inflammation. This combination of effects might explain the effect of methimazole, a much less active compound currently used therapeutically, on suppressing experimental systemic lupus, uveitis, and blepharitis (23–25) or human psoriasis (22). Thus, these findings suggest that the therapeutic effect of methimazole might be due in part to the suppression of ECAM expression and consequent reduction of leukocyte adhesion to the endothelium. Indeed, Graves' patients treated with methimazole have reduced levels of circulating soluble E-selectin and VCAM-1 (31).

The results of the promoter activity assays (Fig. 4) warrant further comment. The increase in TNF-α-induced VCAM-1 promoter activity was not significantly altered by deleting the AP-1 site between -1641 and -288 bp (Fig. 4). This is consistent with previous promoter assays (8) and a report indicating that the AP-1 effect is mediated through the NF-κB element (40). Interestingly, deletion of the region between -288 to -228 bp (which contains the GATA binding sites) did not alter the TNF-α-induced VCAM-1 promoter activity (Fig. 4). This finding is surprising given previous studies with HUVEC that have demonstrated the importance of GATA binding sites in TNF-α-induced VCAM-1 expression (41). We have confirmed our present data using mink lung cells, which are significantly easier to transfect (data not shown). It is possible that as yet undefined *trans* factors and *cis* elements in this region impact GATA activity positively or negatively and that these are differently regulated in HUVEC and HAEC cells. In this respect it was also initially surprising to us that the TNF-α-induced increase in VCAM-1 promoter activity decreased between the $-228/+12$ and $-85/+12$ bp constructs despite the fact both NF-κB elements are intact in the $-85/+12$ bp constructs (Fig. 4). One possible explanation for this finding is that the region between -228 and -85 bp contains yet to be identified important transcriptional regulatory/enhancer elements for TNF-α-induced VCAM-1 expression in HAEC. Additional experiments (e.g., site-directed mutagenesis) are warranted to probe these interesting findings. Finally, the importance of IRF-1 on VCAM-1 promoter activity is consistent with earlier reports (10).

In conclusion, we have found that phenyl methimazole 1) dramatically inhibits TNF-α-induced VCAM-1 expression, has a modest inhibitory effect on E-selectin expression, and has no effect on ICAM-1 expression on HAEC, 2) significantly reduces TNF-α-induced monocytic (U937) cell adhesion to HAEC under in vitro flow conditions similar to that present in vivo, 3) inhibits TNF-α-induced IRF-1 binding activity to VCAM-1 promoter, and 4) reduces TNF-α-induced IRF-1 expression in HAEC. Thus, phenyl methimazole holds promise as a therapeutic for the treatment of pathological inflammation, in particular diseases involving VCAM-1 (e.g., atherosclerosis and inflammatory bowel disease).

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