Iron Chelators Inhibit VCAM-1 Expression in Human Dermal Microvascular Endothelial Cells

Sang-Wahn Koo,* Katherine A. Casper,* Kristen B. Otto,* Amy K. Gira* and Robert A. Swerlick*§
*Department of Dermatology, Emory University School of Medicine, §Department of Veteran Affairs, Dermatology, Decatur Georgia, USA

Vascular cell adhesion molecule (VCAM)-1 expression may be coupled to redox-sensitive regulatory pathways, and iron may play a role in generation of reactive oxygen species that participate in these signaling pathways. To investigate the role of iron in TNFα-induced VCAM-1 gene expression, human dermal microvascular endothelial cells (HDMEC) were stimulated with TNFα in the presence of iron chelators and examined for expression of VCAM-1. The iron chelators dipyridyl (DP) and desferoxamine (DFO) inhibited VCAM-1 protein and mRNA induction in a concentration- and time-dependent manner. The induction of VCAM-1 was not inhibited by nonmetal binding reactive oxygen species (ROS) scavengers, implying a direct effect of iron in the expression of these adhesion molecules. The effect of iron was mediated at the level of gene transcription since pretreatment with DP abrogated the TNFα-mediated up-regulation of VCAM-1 heterogeneous nuclear RNA. Pretreatment of HDMEC with DP prior to TNFα treatment had no effect on p65 nuclear localization, DNA binding, or serine phosphorylation. DP pretreatment inhibited TNFα- and IFNγ-mediated interferon regulatory factor 1 (IRF-1) protein expression, although restoration of IRF-1 expression failed to reconstitute VCAM-1 expression. DP treatment also blocked VCAM-1 induction in human umbilical vein endothelium and blocked induction of a host of NF-kB activated genes in HDMEC including ICAM-1, IL-8, and tissue factor. IkBα, an NF-kB inducible and constitutively accessible gene not requiring chromatin remodeling for transcription, was not affected by DP treatment. These data suggest that iron plays a critical role in TNFα mediated VCAM-1 induction in HDMEC, and the target for iron effects may be IRF-1, NF-kB, and potentially chromatin remodeling.
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Vascular cell adhesion molecule 1 (VCAM-1) is an inducible adhesion protein that mediates heterotypic adhesive events within cells expressing the α4β1 (VLA-4) integrin receptor (Elices *et al*, 1990). VCAM-1 is minimally expressed in endothelial cells (EC) in normal skin. However, VCAM-1 is induced in injury or in a variety of cutaneous inflammatory disorders including psoriasis, atopic dermatitis, delayed type hypersensitivity reactions, late phase reactions, and graft vs. host disease (Norris *et al*, 1991; Norton and Sloane, 1991; Groves *et al*, 1993; Das *et al*, 1994; Onuma, 1994; Wakita *et al*, 1994; Litchfield *et al*, 1996). In experimental models of cutaneous inflammation, blockade of the VCAM-1/VLA-4 binding pathway resulted in diminution of cutaneous inflammatory responses, further supporting an important functional role of VCAM-1 (Silber *et al*, 1994).

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Abbreviations: DFO, desferoxamine; DMPO, 5 dimethyl-1-pyrroline n-oxide; DP, 2,2-dipyridyl; EC, endothelial cell; HUVEC, human umbilical vein endothelial cells; HDMEC, human dermal microvascular endothelial cells; IL-8, interleukin-8; IRF-1, interferon regularory factor 1; PCR, polymerase chain reaction; ROS, reactive oxygen species; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; VCAM, vascular cell adhesion molecule.

Reprint requests to: Dr Robert A. Swerlick, Department of Dermatology, Emory University School of Medicine, WMB 5014, Atlanta, GA 30322, USA; E-mail: rswerli@emory.edu

The induction of VCAM-1 by pro-inflammatory cytokines can be blocked by a variety of agents with antioxidant activity (Marui *et al*, 1993). The mechanism of this effect is not fully defined, although evidence points to involvement of redox- regulation of the activity of transcription factors such as NF-kB. The modulation of redox-sensitive protein kinases and transcription factors by reactive oxygen species (ROS) has previously been suggested to be a central and early event in the induction of inflammatory reactions (Baeuerle and Henkel, 1994). Oxidative stress has also been linked to cutaneous inflammatory disorders such as psoriasis, contact dermatitis and ultraviolet-induced skin changes (Sharkey *et al*, 1991; Lontz *et al*, 1995; Brenneisen *et al*, 1998). In addition, ROS released by inflammatory cells and keratinocytes have been suggested to serve as second messengers in redox-sensitive signal transduction processes (Allen and Tresini, 2000).

Iron, like oxygen, is both physiologically essential and biochemically dangerous (McCord, 1998). Within the body, iron is tightly bound and regulated. Like other transition metals, iron has the capacity to act as a catalyst generating reactive oxygen species that may damage biologic macromolecules. Certain stimuli that induce cell and tissue damage are known to release iron from their normal protective carriers. For example, UV light induced inflammation and DNA damage may be dependent upon UV-mediated release of iron from carrier proteins such as ferritin (Pourzand *et al*, 1999). Like other transition metals, iron also has the capacity to act as a catalyst, generating reactive oxygen species that in turn damage biologic macromolecules. Iron may exert

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biological effects via generation of ROS such as the highly toxic hydroxyl radical ($HO\cdot$), which may then act as an intracellular second messenger (Schreck *et al*, 1991). One potential target for hydroxyl radicals is activation of NF-kB (Schreck *et al*, 1991).

This study was undertaken to address the question of whether iron, via effects on NF-kB activation, plays an important role in the cytokine induction of VCAM-1 in dermal microvascular endothelial cells. We have determined that removal of iron inhibits TNFα-mediated induction of VCAM-1 expression in dermal endothelial cells; this inhibition was not mediated by effects on NF-kB nuclear translocation or DNA binding, but was associated with decreased VCAM-1 gene transcription.

MATERIALS AND METHODS

Cell culture Both primary cultures of human dermal microvascular endothelial cells (HDMEC), obtained from Emory Skin Diseases Research Center, and HDMEC immortalized with SV40 Large T (5A32 cells) were utilized. Cells were isolated and passaged as described previously (Ades *et al*, 1992; Swerlick *et al*, 1992a). Cells were grown in flasks coated with 0.1% gelatin in MCDB 131 media (Mediatech, Herndon, VA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, Utah), 1% penicillin/streptomycin (Mediatech), 2 mM L-glutamine (Mediatech), 0.25 mg/ml cAMP and 1 μg/ml hydrocortisone (Sigma, St. Louis, MO) and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Reagents and antibodies The iron chelators, 2,2-dipyridyl (DP), a soluble chelator of ferrous iron (Fe^{2+}), and desferoxamine (DFO), which binds ferric iron (Fe^{3+}), were obtained from Sigma. 5, 5 dimethyl-1-pyrroline n-oxide (DMPO), a spin trapper without metal binding capability, and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), a nitroxide spin trap that can maintain iron as Fe^{3+}, were also purchased from Sigma. Recombinant human TNFα and IFNγ were purchased from R & D Systems, Inc. (Minneapolis, MN). Anti-IRF-1 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and rabbit polyclonal antip65 and antip50 antibodies were purchased from Biodesign International (Saco, ME). Anti-phospho-p65 (Ser 536) antibody was purchased from Cell Signaling Technology, Inc (Beverly, MA). The VCAM-1 P8B1 monoclonal antibody developed by E.A. Wayner and T. LeBien was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa.

Cell surface ELISA ELISA using anti-VCAM-1 monoclonal antibodies P3C4 (generous gift of Dr. Elizabeth Wayner) or derived from the VII-6G10 hybridoma (ATCC, Rockville, MD), or using anti-ICAM-1 monoclonal antibody 84H10 (generous gift from Dr. Stephen Shaw) was performed as previously described (Ades *et al*, 1992; Swerlick *et al*, 1992b). The cells were treated with DP at doses ranging from 125 to 1000 μM for 0–24 h followed by 16 h of TNFα. Briefly, the cells were incubated with primary antibodies for one hour, fixed with 2% formaldehyde, and washed with Hanks buffer (with calcium and magnesium). Secondary antibody (goat antimouse HRP conjugate, Bio-Rad, Hercules, CA) was incubated for one hour and then washed thoroughly with Hanks buffer (with calcium and magnesium). Color was developed using DAKO TMB solution for 5–30 min, the reaction stopped, and then the plate read with an ELISA reader (Microplate Autoreader Bio-Tek Instruments EL 311 s) at O.D. 450 nm. Figures represent one representative experiment, and all were repeated at least three times. Bars represent the mean OD of four wells ± SD.

Flow cytometric analysis Cell surface expression of VCAM-1 was examined in HDMEC by flow cytometric analysis as previously described (Swerlick *et al*, 1992b). Briefly, HDMEC were stained for VCAM-1 expression before and after TNFα (1000 μ/ml) for 16 h. Cells were trypsinized, aliquotted, pelleted and stained with anti VCAM-1 monoclonal antibody (P1B8, Hybridoma databank) followed by FITC conjugated goat antimouse IgG antibody (Caltag, Burlingame, CA). Mean channel fluorescence was determined for each data point using a minimal of 10000 events. Cell viability was determined by simultaneous staining with propidium iodide.

Measurement of VCAM-1 mRNA and heterogeneous nuclear VCAM-1 RNA (hnRNA) expression HDMEC were treated with 125–500 μM DP for 24 h followed by 200 U/ml TNFα for 24 h. Total RNA was isolated with TriReagent (Sigma) from 5A32 HDMEC according to manufacturer's instructions. cDNA was prepared using the Superscript Preamplification System for 1st Strand cDNA Synthesis (Gibco/BRL, Carlsbad, CA) using the random primer protocol. Real-time polymerase chain reaction (PCR) was done using SYBR green technology with Perkin-Elmer 5700 Gene-Amp Detection System with VCAM-1 primers (forward sequence 5′-CAT GGA ATT CGA ACC CAA ACA-3′ and reverse sequence 5′-GGC TGA CCA AGA CGG TTG TATC-3′). Data was normalized with primers for a housekeeping gene. Relative quantities were determined by generating a standard curve from dilutions of cDNA containing the message of interest. Data was then expressed as fold increase over control. Experiments were repeated at least three times, and data shown are a single representative study.

VCAM-1 hnRNA expression was measured to assess the effect of iron chelation on the transcription of the VCAM-1 gene using a modification of the above technique (Elferink and Reiners, 1996). Prior to reverse transcription, total RNA was treated with RNAse-free DNAse for 1 h at room temperature to destroy any potential contaminating genomic DNA. DNAse was inactivated by heating at 75°C for 20 min VCAM-1 primers were selected that would amplify only nascent, unspliced hnRNA (forward sequence 5′-TAC AGT GTT ACT TCT TCT TCC ACA TTC AA-3′ located at the intron between exon 6 and 7 and reverse sequence 5′-GCC TCA TGA CTC ACT TTA ACC AATT-3′ located at the intron between exon 6 and 7). Emory University Microchemical Facility synthesized all primers.

Electrophoretic mobility-shift assay (EMSA) Confluent 5A32 HDMEC were treated with 500 U/ml TNFα for 2 h after preincubation with 500 μM DP for 24 h. Nuclear proteins were extracted and EMSA performed as described previously (Gille *et al*, 1996). The VCAM-1 oligonucleotide was synthesized to encompass the two NF-kB-like sites of the human VCAM-1 promoter. The double stranded oligonucleotide was 5′-end labeled using the forward reaction with T4 polynucleotide kinase (Gibco/BRL). The DNA binding reaction was performed under the conditions described previously (Gille *et al*, 1996).

The specificity of NF-kB binding complex was further resolved using competition with a cold unlabeled VCAM-kB oligonucleotide, antibodies recognizing p50, p65 (Biodesign International, Saco, ME), or phosphoserine (Calbiochem, San Diego, CA). Competitor and antibodies were added 20 min before adding the radiolabeled probe to the binding reaction. Samples were subjected to electrophoresis on a native 4% polyacrylamide gel for 4 h at 120 V. Experiments were repeated at least three times, and data shown are a single representative study.

Western blot Cells were washed with PBS twice and whole cell extracts were prepared by lysing cells in 50 mM, Tris pH 8.0, 150 mM NaCl, 0.02% Na Azide, 1 μg/ml Aprotinin, 1% Triton X, 1 mM PMSF. NaF was added for the phosphoserine studies (Woetmann *et al*, 1999). The suspension was centrifuged at 12,000 rpm for 15 min at 4°C, and the protein in the supernatant was quantified using the BioRad DC Protein Assay. Whole cell protein extract was resolved with SDS-PAGE using a 10% polyacrylamide gel and reducing conditions. After transfer to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech), the protein was stained with Ponceau S (Sigma) to verify uniform loading and transfer. Membranes were blocked with 5% BSA in TBS-T (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 0.1% Tween 20, pH 7.4) overnight and subsequently incubated with primary antibodies (IRF-1 Ab 1:500, anti-p65 Ab 1:2000, anti-phosphoserine 536 p65 Ab 1:1000) for 1h at room temperature. The membrane was washed with TBS-T three times and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (ECL Reagent, Amersham Pharmacia Biotech).

Immunofluorescence microscopy All cells were grown overnight on eight-well chamber slides (Nalge Nunc International, Naperville, IL) before treatment. Designated wells were left untreated or treated with DP for 24 h, DP followed by stimulation with TNFα 500 U/ml for 30 min, or TNFα alone. The cells were washed with PBS twice for 5 min each and fixed with 3.7% para-formaldehyde for 20 min at room temperature. The cells were washed again and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. The cells were washed and blocked with PBS with 10% FBS at room temperature for at least 10 min. After blocking, the cells were incubated with anti-p65 rabbit antibody (Biodesign) at 1:300 for 1 h at room temperature. The cells were washed three times with blocking solution before adding goat antirabbit FITC conjugated (Caltag, Burlingame, CA) secondary antibody to each well and incubating for 1 h at room temperature in the dark. The cells were washed three times with PBS, mounting reagent added, and the slides visualized with fluorescent microscopy using a Leica DMR-E fluorescence microscope equipped with a Hammamatsu Orca camera. Images were captured and processed using Open Lab software (Improvision Inc., Lexington, MA).

RESULTS

Iron chelators inhibit VCAM-1 induction in HDMEC When endothelial cells were preincubated with 2,2-dipyridyl (DP), a ferrous iron chelator, and then stimulated with TNFα at a dose and for a duration which had been shown previously to maximally induce VCAM-1 expression in HDMEC (Ades *et al*, 1992; Swerlick *et al*, 1992b), VCAM-1 protein expression was inhibited in a concentration- and time-dependent manner. Maximal inhibition was observed at doses as low as 500 μM (**Fig 1*A***). Partial inhibition was observed when DP was added concurrently with TNFα stimulation, with increasing inhibition observed after 8 h (>70%), and complete inhibition requiring at least a 24-h preincubation (**Fig 1*B***). Identical inhibition was observed when expression of induced VCAM-1 was examined by flow cytometric analysis (**Fig 1*C***). No significant decreases in cell viability were noted, even with treatment of cells up to 24 h with doses of DP up to 4 mM when examined by flow cytometric analysis or trypan blue exclusion (data not shown).

In order to address whether the DP-mediated inhibition of VCAM-1 induction was mediated via iron chelation, we examined the effect of a different iron-specific chelator, desferoxamine (DFO), on TNFα-mediated induction of VCAM-1. VCAM-1 protein expression was again inhibited in a concentration-dependent fashion (**Fig 2*A***) with 50% inhibition of TNFα-induced VCAM-1 expression seen at 500 μM, and maximal inhibition noted at 2000 μM. DFO is less cell-permeable than DP, which probably accounts for the need for higher doses for comparable effects. When exogenous iron in the form of ferric citrate (2000 μM) was combined with DFO prior to preincubation, inhibition of TNFα-mediated VCAM-1 induction was partially reversed (**Fig 2*B***). In addition, when 5A32 HDMEC were treated with TEMPO, a nitroxide spin trap that maintains iron as Fe^{3+} (Voest *et al*, 1993), and then stimulated with TNFα, VCAM-1 induction was also inhibited in a concentration-dependent fashion (**Fig 2*D***). However, DMPO, a spin trap without metal binding capability (Rosen and Rauckman, 1984) did not inhibit TNFα induced VCAM-1 up-regulation (**Fig 2*C***). These results further support a role for iron in expression of VCAM-1.

Inhibition of VCAM-1 induction is not cell- or gene-specific To determine whether the DP mediated inhibition was gene or cell-specific, we examined the effect of DP pretreatment on TNFα mediated VCAM-1 induction in human umbilical vein endothelial cells (HUVEC) and on TNFα mediated ICAM-1 induction in HDMEC. DP inhibited VCAM-1 induction of VCAM-1 in HUVEC in a dose dependent manner virtually identical to that observed in HDMEC. Unlike the case for VCAM-1 expression, 8 h of pretreatment with DP (500 μM) had virtually no effect on ICAM-1 cell surface expression. However, preincubation of cells with DP for 24 h resulted in marked inhibition of ICAM-1 protein expression (**Fig 3**).

Iron chelators inhibit VCAM-1 gene transcription In order to examine whether inhibition of VCAM-1 expression may be mediated by changes in gene expression, we initially examined whether DP pretreatment prior to TNFα stimulation inhibited TNFα-mediated increases in VCAM-1 mRNA expression. DP pretreatment inhibited up-regulation of VCAM-1 mRNA by >90% (**Fig 4*A***). Similarly, DFO almost completely inhibited TNFα-mediated increases in VCAM-1 mRNA levels (**Fig 4*B***). In contrast, the nonmetal binding spin trap DMPO had no effect, consistent with its lack of effect on TNFα induced VCAM-1 protein expression (**Fig 4*B***).

In order to directly examine whether iron chelators inhibit VCAM-1 expression by inhibition of VCAM-1 gene transcription, we utilized real time PCR to measure the expression of VCAM-1 hnRNA after cytokine stimulation in the presence and absence of the iron chelator DP. PCR primer pairs were designed that recognized intronic sequences that are found only in unspliced VCAM-1 hnRNA. Treatment of cells with TNFα resulted in a marked induction of hnRNA that was inhibited by pretreatment with actinomycin D or DP (**Fig 5**). These data provide support for VCAM-1 gene transcription as being the target for iron chelator mediated inhibition of VCAM-1 expression.

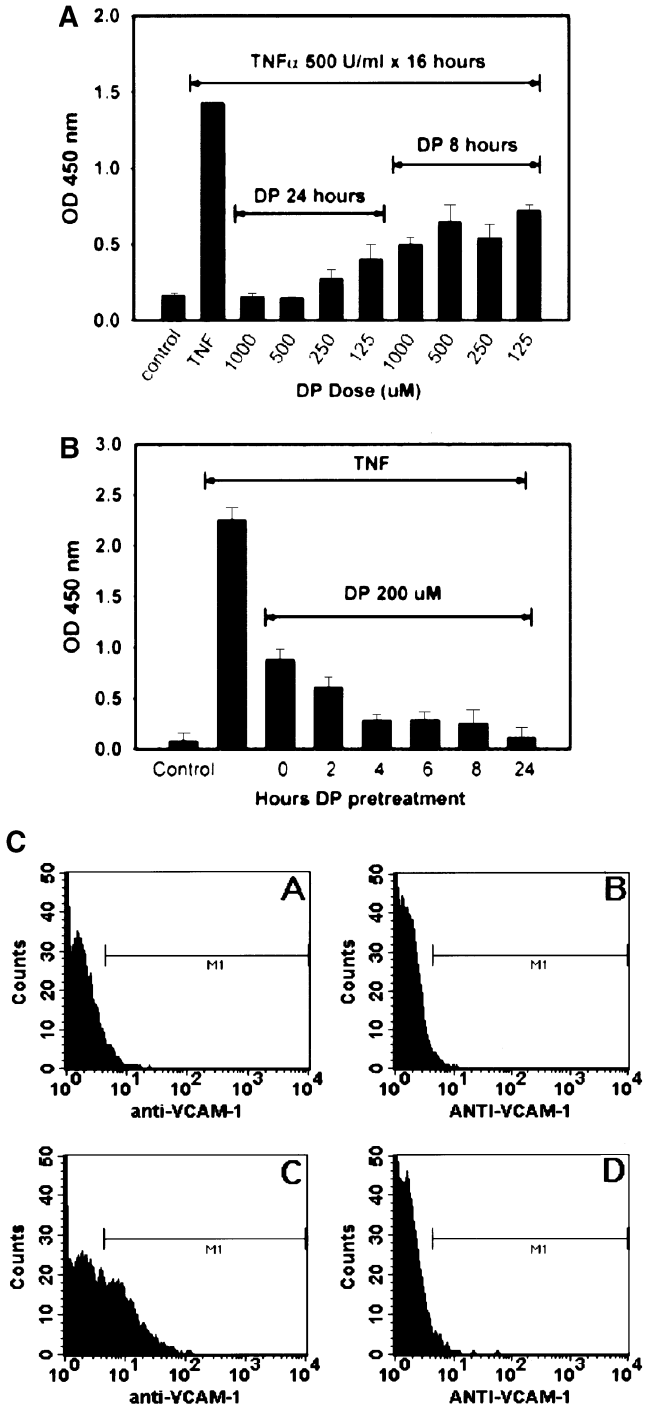

Figure 1. DP pretreatment blocks TNFα-mediated VCAM-1 induction in 5A32 HDMEC in a dose and time-dependent manner. HDMEC were pretreated with DP before stimulation with TNFα and cell-surface VCAM-1 measured by ELISA. (*A*) 5A32 HDMEC were pretreated with increasing doses of DP for 8 or 24 h as indicated followed by 500 U/ml TNFα for 16 h. (*B*) 5A32 HDMEC were pretreated with 500 μM DP for times ranging from 0 to 24 h and then stimulated with 500 U/ml TNFα for 16 h. (*C*) Flow cytometric analysis of 5A32 HDMEC: Untreated (*A*), DP 1000 μM × 24 h (*B*), TNFα 1000 U/ml × 16 h (*C*), and DP treated (1000 μM × 24 h) followed by TNFα (1000 U/ml × 16 h).

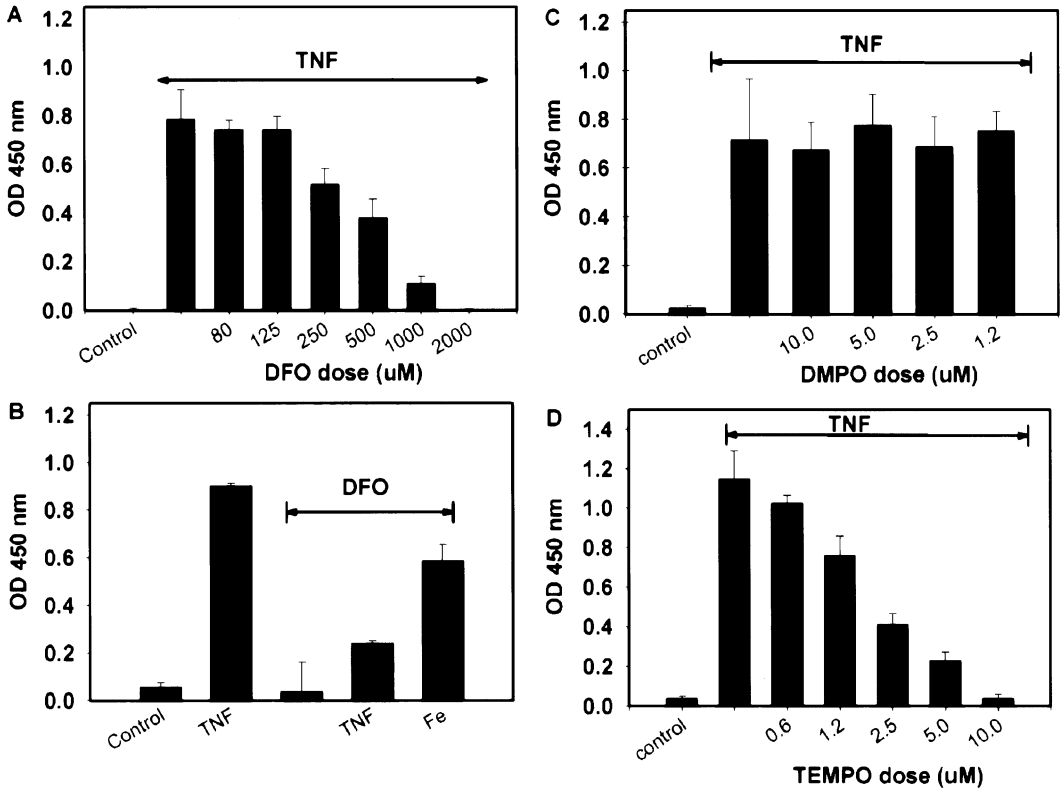

Figure 2. Iron chelation activity is required for VCAM-1 blockade. (*A*) 5A32 cells were pretreated with increasing concentrations of the iron chelator DFO for 4 h prior to stimulation with TNFα (100 U/ml × 16 h). Cell surface VCAM-1 expression was measured by ELISA. (*B*) DFO- mediated inhibition of VCAM-1 induction was partially reversed when cells were pretreated with 200 μM DFO plus ferric citrate (2000 μM × 4 h) and stimulated with TNFα (500 U/ml × 16 h). 5A32 HDMEC were pretreated with the indicated concentrations of DMPO (*C*) and TEMPO (*D*) for 2 h and were stimulated with TNFα (500 U/ml × 16 h). TNFα-mediated VCAM-1 expression was blocked in a dose-dependent manner by TEMPO, a metal binding spin trapper that can maintain iron as Fe^{3+}, but not by DMPO, a nonmetal binding spin trapper, providing further support for a role of iron in VCAM-1 expression in 5A32 HDMEC.

Iron chelators do not inhibit NF-kB translocation or DNA binding Iron chelators have been shown to regulate the stability of transcription factors (Wang and Semenza, 1993; Lin *et al*, 1997). Previous studies have also demonstrated that certain antioxidants may also block translocation of activated NF-kB complexes from the cytoplasm to the nucleus, thus inhibiting NF-kB mediated gene activation (Bowie *et al*, 1997). We hypothesized that iron chelator-mediated inhibition of VCAM-1 induction may be controlled via effects on NF-kB stability or function. In order to test this hypothesis, we examined nuclear localization of NF-kB complexes after TNFα stimulation using immunofluorescence microscopy. TNFα stimulation of HDMEC resulted in prompt translocation of p65 from the cytoplasm to the nucleus. However, DP pretreatment prior to TNFα had no detectable effect on this TNFα-mediated p65 translocation (**Fig 6**).

It was possible that although DP pretreatment may not affect NF-kB translocation, DP may alter the ability of translocated complexes to bind to relevant sequences in the VCAM-1 promoter. In order to examine whether DP pretreatment resulted in loss of NF-kB binding activity despite apparently normal translocation, nuclear extracts were examined for protein binding to the VCAM-1 NF-kB sequence using EMSA (**Fig 7**). DP treatment had no effect on the binding of translocated NF-kB complexes. After TNFα stimulation (***lanes 3–8***), complex formation was the same in both DP pretreated (***lanes 6–8***) and untreated samples (***lanes 3–5***). New complex formation (***bands A and B, lanes 3 and 6***), which was shifted with addition of antip50 (***band C, lanes 4 and 7***) or antip65 (***band D, lanes 5 and 8***) antibodies was also identical in each case. These data would suggest that the DP effect is mediated via targets other than NF-kB translocation or DNA binding.

Iron chelators inhibit TNFα-mediated IRF-1 induction The previous data suggested that the effect of DP on VCAM-1 gene transcription may be mediated via an NF-kB independent mechanism. Another potential target is interferon regulatory factor 1 (IRF-1), a transcription factor that is also induced by TNFα. In addition to the tandem NF-kB elements in the VCAM-1 promoter, optimal transcriptional induction of VCAM-1 depends to some degree upon binding of IRF-1 to a distinct and adjacent element in the VCAM-1 promoter (Neish *et al*, 1995; Lechleitner *et al*, 1998). In order to examine whether iron chelation blocks IRF-1 expression, we examined IRF-1 protein expression after cytokine stimulation with and without DP pretreatment. Treatment of HDMEC with either TNFα or IFNγ induced expression of IRF-1 protein. Pretreatment of 5A32 HDMEC with DP prior to either TNFα or IFNγ treatment completely inhibited the expression of IRF-1 protein expression. This inhibition was dependent upon DP concentration and preincubation time, roughly paralleling DP effects on VCAM-1 induction. Thus, DP may inhibit VCAM-1 induction via inhibition of IRF-1 induction (**Fig 8*A***).

In order to examine whether the DP mediated inhibition of VCAM-1 induction was primarily mediated via effects on IRF-1 expression, we preinduced IRF-1 expression in 5A32 cells with IFNγ. By doing so, we were able to induce persistent IRF-1 expression without inducing VCAM-1 and IRF-1 expression persisted for up 8 h, even after the addition of DP. HDMEC were then treated with TNFα, with and without DP pretreatment (**Fig 8*B***). We then utilized cell based ELISA to determine whether preinduction of IRF-1 by IFNγ could restore VCAM-1 induction by TNFα. However, induction of IRF-1 with IFNγ prior to DP treatment only partially restored TNFα-mediated VCAM-1 induction (**Fig 8*C***). These data

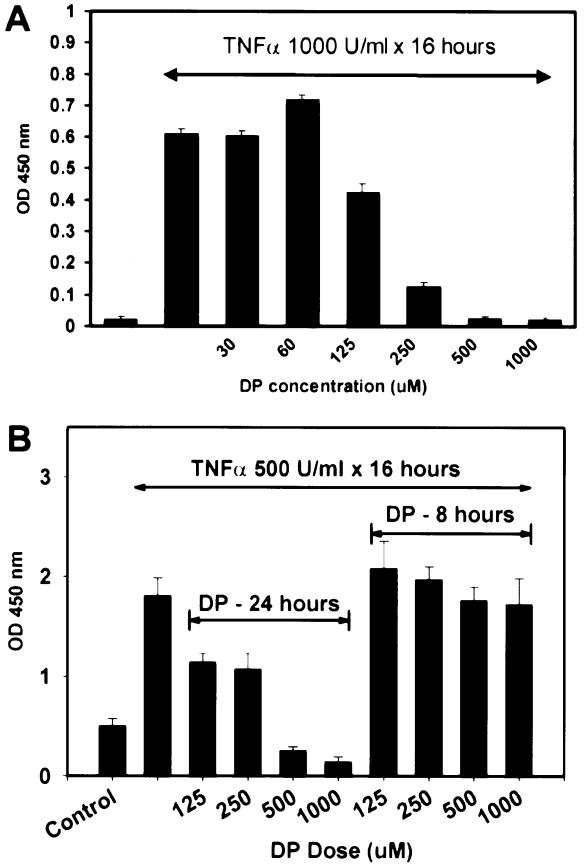

Figure 3. DP mediated inhibition is not cell- or gene specific: (*A*) HUVEC were pretreated with DP (30–1000 μM) prior to stimulation with TNFα (1000 U/ml × 16 h). Cells were assayed for VCAM-1 expression by cell based ELISA. (*B*) 5A32 HDMEC were pretreated with various concentrations of DP for 8 or 24 h followed by 500 U/ml TNFα for 16 h. Cell-surface levels of ICAM-1 were measured by ELISA. Preincubation of cells with DP for 24 h prior to cytokine treatment significantly blocked TNFα-mediated ICAM-1 induction.

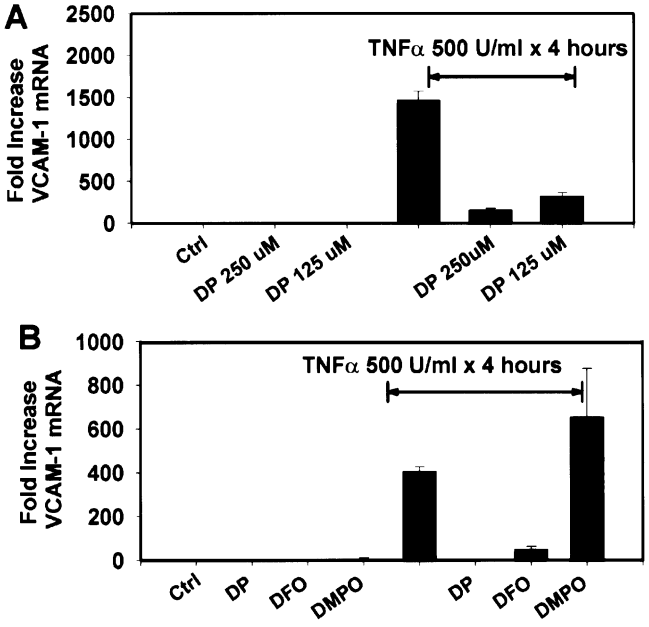

Figure 4. DP and DFO but not DMPO inhibits TNFα-mediated VCAM-1 mRNA up-regulation. (*A*) 5A32 HDMEC were pretreated with 250 or 125 μM DP for 24 h and then stimulated with 200 U/ml TNFα for 24 h. VCAM-1 mRNA was measured by quantitative RT-PCR and differences represented as fold increase over baseline. (*B*) Confluent monolayers of 5A32 HDMEC were pretreated with 500 μM DP, 500 μM DFO, or 500 μM DMPO for 24 h and then stimulated with 200 U/ml TNFα for 24 h. DP and DFO inhibit TNFα-mediated increases in VCAM-1 mRNA, but DMPO did not.

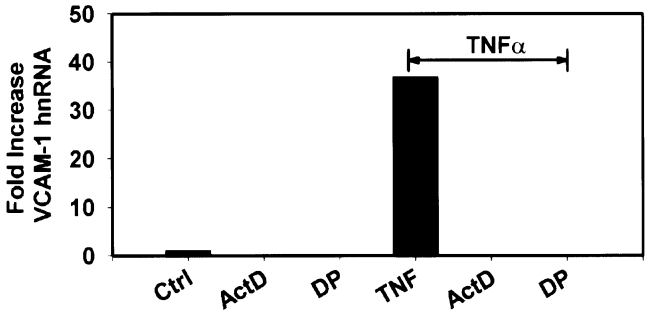

Figure 5. DP inhibits TNFα-mediated VCAM-1 expression by inhibition of VCAM-1 gene transcription. 5A32 HDMEC were pretreated with 500 μM DP for 8 h or 2 μg/ml actinomycin D for 30 min prior to stimulation with 500 U/ml TNFα for 4 h. The fold differences of VCAM-1 hnRNA were determined by quantitative RT-PCR. Results are representative of three experiments.

suggest that although inhibition of IRF-1 expression by DP may play a partial role in DP mediated-VCAM-1 inhibition additional targets for DP effect needed to be defined.

Iron chelators do not inhibit NF-kB phosphorylation Previous studies have suggested that changes in NF-kB phosphorylation, particularly at serine residues, may be critical for the ability of NF-kB proteins, particularly p65, to transactivate genes without affecting DNA binding (Wang and Baldwin, 1998; Wang *et al*, 2000). To examine whether DP pretreatment inhibited p65 phosphorylation, we utilized antibody in western analysis that specifically recognized serine-phosphorylated p65. Under basal conditions, some p65 was constitutively phosphorylated and TNFα stimulation resulted in a prompt increase in the level of phosphorylation. Pretreatment of HDMEC with DP had no effect on constitutive phosphorylation or the increases associated with TNFα treatment (**Fig 9**).

Iron chelators inhibit cytokine induction of NF-kB genes with delayed kinetics, but not IkBα Despite blocking the TNFα mediated induction of VCAM-1 and ICAM-1, DP appeared to have not discernable effect on NF-kB translocation, binding, or phosphorylation that might affect its transactivating function. Alternative targets requiring iron are mechanisms regulating chromatin structure and availability of promoters of NF-kB responsive genes. Previous studies have shown that genes that are rapidly activated after cytokine stimulation, such as IkBα, do not require chromatin modification for transcription factor accessibility (Saccani *et al*, 2001; Saccani *et al*, 2002). In order to examine whether DP may affect VCAM-1 expression via this target, we examined the TNFα induction of both genes with constitutively and immediately accessible (CIA) promoters (IkBα) and those with delayed inductions that presumably require stimulus-dependent modifications in chromatin structure to make NF-kB sites accessible (Saccani *et al*, 2001; Saccani *et al*, 2002). DP pretreatment blocked induction of mRNA of two additional NF-kB regulated genes, interleukin-8 (**Fig 10**) and tissue factor (data not shown), in HDMEC. In contrast, DP had no effect on the TNFα mediated up-regulation of IkBα mRNA (**Fig 10**). These data are consistent with DP targeting cytokine-mediated effects on chromatin packaging.

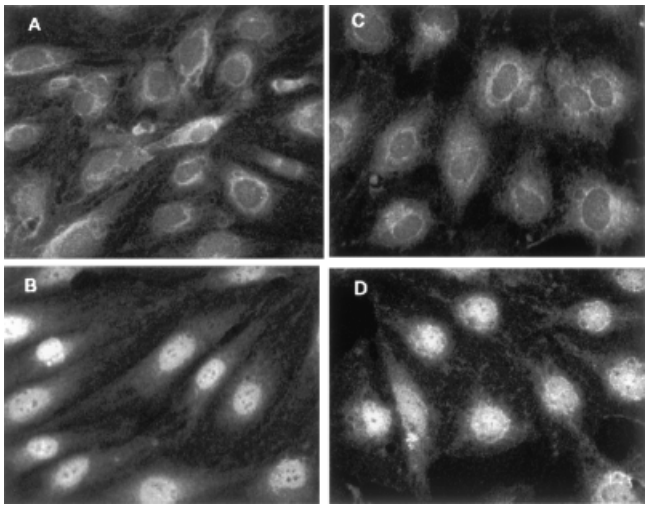

Figure 6. DP does not affect NF-kB translocation after TNFα stimulation in 5A32 HDMEC cells. (*A*) Untreated cells; or (*B*) treated with 500 U/ml TNFα for 30 min; (*C*) 500 μM DP for 24 h; and (*D*) 500 U/ml TNFα for 30 min after pretreatment with DP 500 μM for 24 h. Cellular localization of p65 was identified by immunofluorescence microscopy.

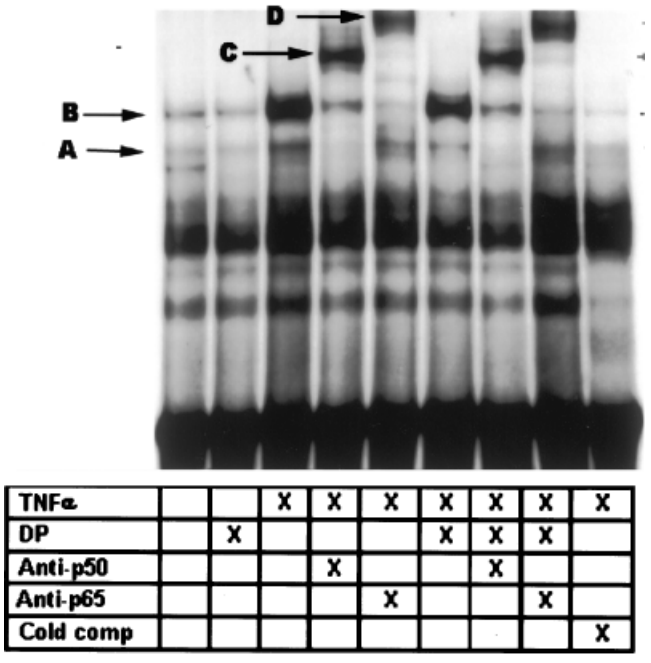

Figure 7. DP does not affect NF-kB binding to the VCAM-1 kB element after TNFα stimulation. 5A32 HDMEC were stimulated with 500 U/ml TNFα for 2 h with or without pretreatment with DP (500 μM) for 24 h. Nuclear extracts were assessed for the binding of NF-kB by EMSA. TNFα treatment results in appearance of complexes A and B, which appear in cells regardless of whether pretreated with DP. In addition, both bands are supershifted in treated cells by antip50 antibody (*band C*) or antip65 antibody (*band D*) with or without DP pretreatment.

DISCUSSION

Iron has been implicated as a key participant in a variety of inflammatory disorders and processes. Studies examining the effect of iron chelators on the function of key transcription factors such as NF-kB and HIF-1α have shown that chelation of iron affects proteasomal processing, resulting in inhibited degradation (Wang

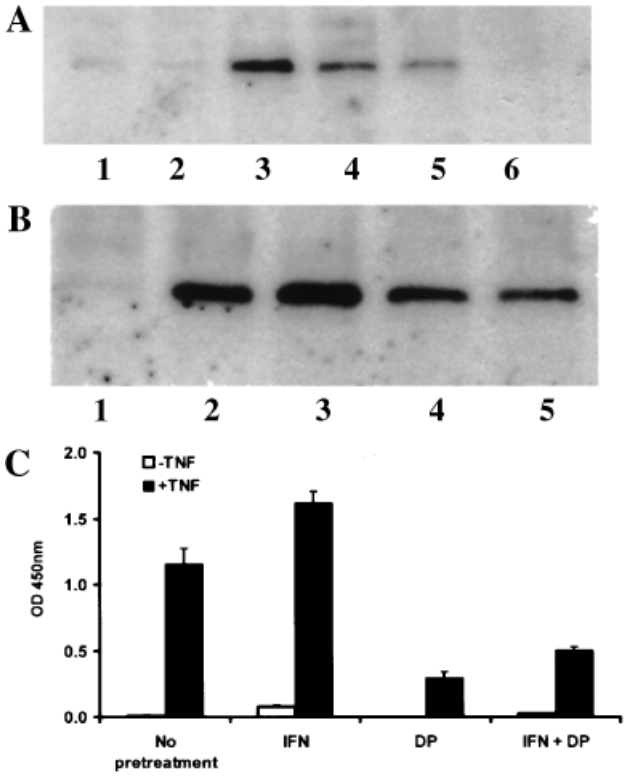

Figure 8. Pretreatment of 5A32 HDMEC cells with IFNγ prior to DP pretreatment induces persistent IRF-1 expression but only partially restores TNFα-mediated VCAM-1 induction. (*A*) 5A32 HDMEC were pretreated for 4, 8 and 24 h with 1000 μM DP prior to TNFα stimulation. Cell lysates were assayed for the presence of IRF-1 by Western blotting. Untreated cells (*lane 1*), DP treated (500 μM × 24 h, *lane 2*), TNFα treated (500 U/ml × 2 h, *lane 3*) or 2 h of TNFα treatment preceded by incubations of DP (500 μM) for 4 h (*lane 4*), 8 h (*lane 5*), and 24 h (*lane 6*). (*B*) IFNγ induces IRF-1 expression that persists after DP treatment. Untreated cells (*lane 1*), IFNγ (1000 U/ml) for 2 h (*lane 2*), IFNγ (1000 U/ml) for 10 h (*lane 3*), IFNγ (1000 U/ml) × 2 h followed by DP (1000 μM) for 8 h (*lane 4*), and IFNγ (1000 U/ml) for 2 h followed by DP (1000 μM) for 24 h (*lane 5*). Cell lysates were prepared and assessed for the presence of IRF-1 by Western blotting. (*C*) Prior induction of IRF-1 by IFNγ does not restore VCAM-1 induction inhibited by DP pretreatment as measured by ELISA. Control cells (untreated), IFNγ (1000 U/ml × 24 h), DP (500 μM × 24 h), IFN/DP (IFNγ 1000 U/ml × 2 h followed by DP 500 μM × 24 h), TNFα 500 U/ml × 16 h), IFN/TNF (IFNγ 1000 U/ml × 2 h then TNFα 500 U/ml × 16 h), DP/TNF (DP 200 μM × 6 h then TNFα 500 U/ml × 16 h), and IFN/DP/TNF (IFNγ 1000 U/ml × 2 h, then DP 500 μM × 6 h, then TNFα 500 U/ml × 16 h). Pretreatment of the cells with IFNγ and induction of IRF-1 expression prior to DP pretreatment only partially restores TNFα-mediated VCAM-1 induction.

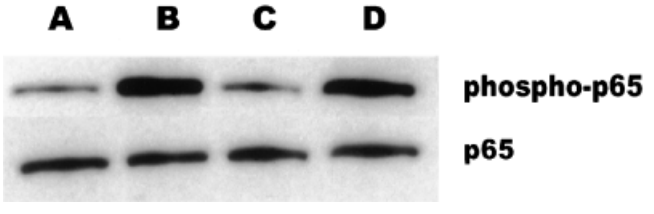

Figure 9. DP does not globally inhibit serine phosphorylation of p65. 5A32 HDMEC cells were pretreated for 20 h with 500 μM of DP prior to stimulation with 500α U/ml TNFα for 30 min Cell lysates (*A*) untreated HDMEC (*B*) TNFα 500 U/ml × 30 min (*C*) DP 500 μM × 24 h (*D*) DP 500 μM × 24 h, then TNFα 500 U/ml × 30 min, were assessed for the presence of phospho-Ser 536 p65 antibody (Cell Signaling Technology) by Western blotting. The same blots were then stripped and probed with antip65 antibody.

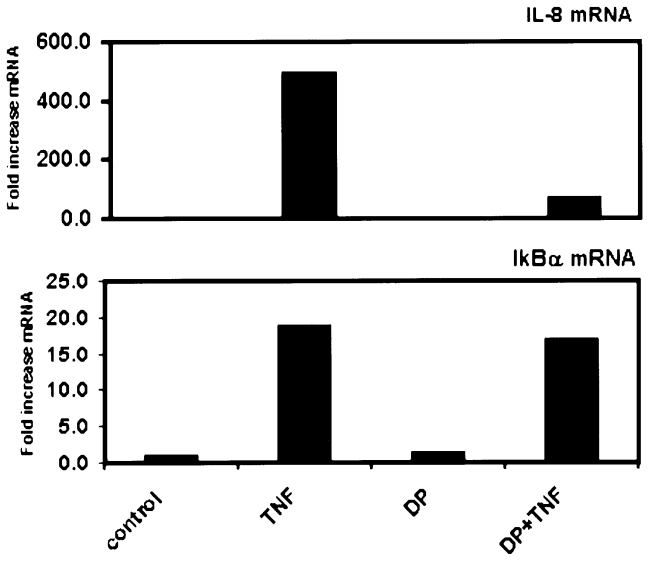

Figure 10. DP blocks TNFα mediated increases in IL-8 mRNA but does not inhibit increases in IkBα mRNA: 5A32 HDMEC were stimulated with TNFα (1000 U/ml × 4 h) with and without pretreatment with DP (1000 μM × 24 h). Steady state levels of IL-8 and IkBα mRNA were assessed by real-time quantitative PCR as described in material and methods. Levels are expressed as fold-increase over unstimulated, untreated controls.

and Semenza, 1993; Bowie *et al*, 1997). Given the dependence of VCAM-1 expression on proteasomal degradation of the IkBα regulatory subunit of NF-kB, it appeared to be reasonable to predict that iron chelators might inhibit VCAM-1 induction by TNFα. Consistent with our hypothesis, the iron chelator DP almost completely inhibited TNFα-mediated induction of VCAM-1 at the protein and mRNA level.

Similar effects upon TNFα-mediated induction of VCAM-1 were also observed in HDMEC with pretreatment using a different iron specific chelator, DFO. Addition of exogenous iron in the form of ferric citrate to DFO before stimulation with TNFα partially reversed the chelator effect, supporting again iron binding as the target of action of iron chelators. The electron spin trap 5-dimethyl-1-pyrrolone-N-oxide (DMPO), which does not bind to iron, failed to inhibit TNFα mediated induction of VCAM-1. However, when HDMEC were treated with TEMPO, a nitroxide spin trap that maintains iron as Fe^{3+}, and then stimulated with TNFα, VCAM-1 induction was inhibited in a dose-dependent fashion. These data further support the role of iron in TNFα-mediated VCAM-1 up-regulation.

Our data clearly point to VCAM-1 gene transcription as a target for DP. DP pretreatment completely blocked TNFα mediated increases in VCAM-1 steady state mRNA expression, and hnRNA induction. However, the exact mechanism of this effect is not completely defined. Iron is required for activity of an intracellular prolyl hydroxylase that modifies HIF-1α, facilitating its interaction with Von Hippel Lindau protein and proteasomal degradation (Ivan *et al*, 2001). Similarly, DFO has also been shown to inhibit TNFα mediated IkBα degradation. We hypothesized that iron chelation may inhibit VCAM-1 gene activation by blocking TNFα-mediated IkBα degradation and NF-kB activation. However, iron chelators had no effect on either NF-kB translocation or the ability of translocated complexes to bind to VCAM-1 kB elements, even at doses sufficient to block VCAM-1 protein and mRNA expression. Given the lack of obvious effect of DP pretreatment on NF-kB binding to the VCAM-1 kB element, other potential targets for iron chelators had to be considered.

VCAM-1 expression has also been shown to be sensitive to antioxidants such as *n*-acetylcysteine (NAC) and pyrrolidine-dithiocarbamate (PDTC) (Marui *et al*, 1993). Agents such as PDTC are also metal chelators, and it has been proposed that their potent effects may be mediated via a combination of metal chelation and related antioxidant effects (Saran and Bors, 1990). It is possible that chelation of iron results in loss of metal dependent redox signaling. However, our data does not support this explanation since antioxidant mediated inhibition of VCAM-1 induction has been shown to be mediated via inhibition of NF-kB activation and translocation, and DP had no effect whatsoever on NF-kB activation, translocation, or DNA binding (Bowie *et al*, 1997).

Alternatively, iron chelators may inhibit VCAM-1 induction by blocking the induction of IRF-1 expression. Previous studies have suggested that although IRF-1 augments the expression of VCAM-1 transcription, it is not necessary for gene expression (Neish *et al*, 1995; Lechleitner *et al*, 1998). Our data are consistent with these previous observations. Pretreatment of endothelial cells with DP completely blocked TNFα-mediated induction of IRF-1 expression. However, prior induction of IRF-1 by treatment with IFNγ only partially restored TNFα-mediated VCAM-1 induction. Furthermore, DP pretreatment blocks TNFα mediated up-regulation of ICAM-1 expression in HDMEC, a process that is NF-kB dependent and IRF-1 independent (Gille *et al*, 1997; Paxton *et al*, 1997).

It is possible that iron is required for NF-kB complexes to transactivate the VCAM-1 gene without affecting binding to sites in the promoter as assessed by EMSA. Previous studies have shown that anti-inflammatory agents such as sulfasalazine inhibit the p65 phosphorylation, and this phosphorylation is critical for p65-mediated gene transactivation (Egan *et al*, 1999). The target for phosphorylation may be one or more of a series of serine residues. Multiple enzymes, which induce phosphorylation of specific serine residues, have been described (Wang and Baldwin, 1998; Wang *et al*, 2000). Our EMSA studies do not demonstrate any global inhibition of NF-kB serine phosphorylation by DP. In addition, iron chelators do not block TNFα-mediated phosphorylation of Ser536, a residue previously identified for its role in p65 function (Sakurai *et al*, 1999). However, our studies cannot rule out an effect on phosphorylation of other residues such as Ser529 (Wang and Baldwin, 1998; Wang *et al*, 2000). Iron may still be critical for the phosphorylation of specific serine residues in p65, and altered patterns of phosphorylation may affect VCAM-1 gene transcription without affecting translocation or DNA binding.

Paradoxically, although DP did not affect the activation of NF-kB or its ability to bind *in vitro* to oligonucleotides corresponding to VCAM-1 kB elements, it still almost completely blocked VCAM-1 gene transcription mediated by TNFα. It is possible that iron blocked VCAM-1 induction in HDMEC via a mechanism completely independent of NF-kB. This is quite unlikely given previous studies of TNFα mediated VCAM-1 gene expression in this context showing a strict dependence on NF-kB (Iademarco *et al*, 1992; Neish *et al*, 1995; Gille *et al*, 1996). An alternative mechanism may be an iron dependent process regulating chromatin modifications and accessibility. The effects of iron chelators on TNFα mediated genes in HDMEC are consistent with an effect on DNA packaging and histone modification. The differential effects on VCAM-1 and IkBα could be explained on the basis of differential requirements for chromatin remodeling as a prerequisite for transcription (Saccani *et al*, 2001). Certain NF-kB driven genes are not immediately accessible and require modification of histones in addition to NF-kB translocation for expression. The transcription of these genes tends to be delayed after cytokine stimulation and NF-kB activation, presumably because histone modification requires additional time. In contrast, other NF-kB driven genes such as IkBα are immediately accessible and are rapidly induced after cytokine stimulation. We reasoned that if iron was important for chromatin accessibility, iron chelators should not have an effect on genes such as IkBα that were constitutively accessible while iron chelators should inhibit expression of NF-kB inducible genes with delayed kinetics. Our observations that DP pretreatment had no effect on IkBα

induction, while consistently blocking other NF-kB inducible genes with delayed kinetics, support this hypothesis.

Previous studies linking chromatin remodeling to iron also provide additional indirect evidence for the role of iron. Iron is essential for expression of a number of genes critical for cell growth and proliferation, and removal of iron via the use of DFO has effects that are similar to the effects of histone deacetylase (HDAC) (Kim *et al*, 2001) (Kramer *et al*, 2002). Treatment of cells with DFO resulted in cell cycle arrest and down-regulation of $p21^{WAF}$ and specific cyclin dependent kinases. From these data, one can conclude that expression of $p21^{WAF}$ is iron dependent (Kramer *et al*, 2002). Similarly, HDAC inhibition resulted in gene expression comparable to iron loading, with resultant expression of $p21^{WAF}$ and cell proliferation (Kim *et al*, 2001). Consistent with our hypothesis is the converse of these observations. Histone deacetylation has effects that are essentially identical to iron chelation. It is reasonable to propose that they may operate via a common mechanism and that mechanism likely involves chromatin accessibility.

In conclusion**,** iron chelators inhibited VCAM-1 protein and mRNA induction in a concentration- and time-dependent manner via a mechanism that appears to be transcriptionally mediated. The target of iron chelator effects was not NF-kB activation, but appeared to be mediated through a combination of inhibition of IRF-1 expression and effects on the ability of translocated NF-kB complexes to transactivate the VCAM-1 gene, potentially via iron dependent alterations in chromatin remodeling.

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