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Hypoxia and hypoxia mimetics inhibit TNF-dependent VCAM1 induction in the 5A32 endothelial cell line via a hypoxia inducible factor dependent mechanism

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ABSTRACT

Background: We previously reported that iron chelators inhibit TNFα-mediated induction of VCAM-1 in human dermal microvascular endothelial cells. We hypothesized that iron chelators mediate inhibition of VCAM-1 via inhibition of iron-dependent enzymes such as those involved with oxygen sensing and that similar inhibition may be observed with agents which simulate hypoxia.
Objective: We proposed to examine whether non-metal binding hypoxia mimetics inhibit TNFα-mediated VCAM-1 induction and define the mechanisms by which they mediate their effects on VCAM-1 expression.
Methods: These studies were undertaken *in vitro* using immortalized dermal endothelial cells, Western blot analysis, ELISA, immunofluorescence microscopy, quantitative real-time PCR, and chromatin immunoprecipitation.
Results: Hypoxia and the non-iron binding hypoxia mimetic dimethyl oxallyl glycine (DMOG) inhibited TNFα-mediated induction of VCAM-1. DMOG inhibition of VCAM-1 was dose-dependent, targeted VCAM-1 gene transcription independent of NF-κB nuclear translocation, and blocked TNFα-mediated chromatin modifications of relevant elements of the VCAM-1 promoter. Combined gene silencing of both HIF-1α and HIF-2α using siRNA led to a partial rescue of VCAM expression in hypoxia mimetic-treated cells.
Conclusion: Iron chelators, non-metal binding hypoxia mimetics, and hypoxia all inhibit TNFα-mediated VCAM-1 expression. Inhibition is mediated independent of nuclear translocation of NF-κB, appears to target TNFα-mediated chromatin modifications, and is at least partially dependent upon HIF expression. The absence of complete VCAM-1 expression rescue with HIF silencing implies an important regulatory role for an Fe(II)/α-ketoglutarate dioxygenase distinct from the prolyl and asparagyl hydroxylases that control HIF function. Identification of this dioxygenase may provide a valuable target for modulating inflammation in human tissues.
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1. Introduction

Cytokine inducible cell adhesion molecules (CAMs) on vascular endothelium comprise a tightly regulated family of cell-surface proteins, which mediate leukocyte adhesion to endothelial cells and subsequent diapedesis into the extravascular tissue compartments, including the skin. One specific vascular CAM, vascular cell adhesion molecule (VCAM)-1 is induced by a limited set of cytokines including TNFα and binds to the α4β1integrin, which is present on non-neutrophilic leukocytes [1].

VCAM-1 is robustly expressed on dermal endothelium in numerous inflammatory skin disorders, including psoriasis, atopic dermatitis, and delayed type hypersensitivity, underscoring its integral role in inflammatory processes [2,3]. Pharmacologic disruption of the α4β1integrin/VCAM-1 interaction blunts inflammatory responses in multiple animal models of cutaneous disease as well as chronic inflammatory states such as rheumatoid arthritis, inflammatory bowel disease, and asthma [4]. The clinical potential of modulating the α4β1integrin/VCAM-1 interaction has been further highlighted by recent clinical trials demonstrating the efficacy of the anti-α4β1integrin monoclonal antibody, natalizu-

Abbreviations: α-KG, α-ketoglutarate; CAM, cell adhesion molecule; DMOG, dimethyl oxallyl glycine; DP, 2,2′-dipyridyl; egr-1, early growth response protein 1; FIH1, factor inhibiting HIF; HDMEC, human dermal endothelial cells; HIF, hypoxia inducible factor(s); hnRNA, heterogenous nuclear RNA; IRF-1, interferon regulatory factor 1; PHD, prolyl hydroxylase; RT-PCR, reverse transcription real-time polymerase chain reaction; TGFα, transforming growth factor-α; VCAM, vascular cell adhesion molecule.

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mab, in the treatment of relapsing multiple sclerosis [5,6] as well as Crohn's disease [7].

Iron chelators potently inhibit TNFα-mediated VCAM-1 protein expression in human dermal endothelial cells (HDMEC) through a marked reduction in VCAM-1 gene transcription [8]. However, iron chelators do not inhibit TNFα-induced nuclear factor κB (NF-κB) activation, nuclear translocation, or the ability of nuclear localized NF-κB complexes to bind to relevant NF-κB binding oligonucleotides, all previously characterized critical regulators of VCAM-1 induction.

One possible target for iron chelators is hypoxia inducible factor (HIF), the central transcription factor implicated in coordinating the cascade of events involved in cellular adaptation to hypoxia [9]. HIF is a heterodimer of a constitutively expressed β subunit and one of three tightly regulated alpha subunits (HIF-1α, 2α, or 3α). HIF-1α and 2α are hypoxia inducible proteins. Under normoxic conditions, prolyl hydroxylases (PHD) hydroxylate both HIF-1α and HIF-2α at specific proline residues enabling ubiquitination and consequent proteasomal degradation. PHDs are oxygen, Fe(II), and α-ketoglutarate (α-KG)-dependent and become enzymatically inactive in the absence of any one of these factors [10]. In the absence of any one of the cofactors unhydroxylated HIF-1α/2α escapes destruction and translocates to the nucleus where it affects transcription of hypoxia responsive genes.

It is possible that iron chelators may alter the function of iron- and oxygen-dependent enzymes by sequestering required iron. We therefore hypothesized that iron chelators may mediate anti-inflammatory effects by a novel pathway, functioning as hypoxia mimetics. In this study, we report the abrogation of TNFα-mediated VCAM-1 expression in HDMEC by both hypoxia and a hypoxia mimetic mechanistically distinct from metal chelators, the α-KG antagonist dimethyl oxallyl glycine (DMOG). These agents appear to target TNFα-mediated VCAM-1 gene transcription via inhibition of chromatin modification. Furthermore, using silencing RNA we demonstrate that HIF isoforms are partially responsible for this phenomenon, affirming a novel link between VCAM-1 expression and the hypoxic cellular response pathways, but also implicating other as yet undefined pathways in the transcriptional regulation of this important inflammatory mediator.

2. Materials and methods

2.1. Cell culture

All studies were done in the 5A32 endothelial cell line [11] derived from primary HDMEC immortalized with SV40 Large T which were subcultured as described previously [12]. HDMEC were cultured under continuous reduced oxygen conditions (1%) using a Coy O_2 Control Isolation Glovebox (Coy Laboratory Products, Grass Lake, MI). Cell viability was confirmed with trypan blue exclusion.

2.2. Reagents and antibodies

DP was obtained from Sigma. DMOG was obtained from Cayman Chemical (Ann Arbor, MI). Recombinant human TNFα and TGFα were purchased from R & D Systems Inc. (Minneapolis, MN). Monoclonal antibodies against VCAM-1 (6G10 or P3C4) and ICAM-1 (P2A4), used in cellular ELISA, were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). For Western blots, Santa Cruz Biotechnology (Santa Cruz, CA) supplied antibodies against VCAM-1 (sc-1809), egr-1 (sc-189), and IRF-1 (sc-497). Novus Biologicals (Littleton, CO) provided antibodies specific for HIF-1α (100–105) and HIF-2α (100–132). Immunocytochemistry involved an anti-p65 antibody (K59165R) from Biodesign International (Saco, Maine).

2.3. Cellular ELISA

Expression of VCAM-1 was assessed using a cell-based ELISA system as previous described [13]. All experiments were performed in triplicate, with data from a representative experiment presented. Error bars reflect the average absorbance of 4 wells ± SD.

2.4. Western blot

Cytosolic and nuclear extracts were prepared with Pierce's NE-PER nuclear extraction kit (Rockford, IL) according to the manufacturer's protocol. Whole-cell extracts were prepared by cell lysis in a single detergent extraction buffer, and all extracts were electrophoresed by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes as previously described [11]. Membranes were blocked for 1 h with TBS–tween/5% non-fat dry milk, and then incubated with primary antibody overnight (egr-1 1:1000, HIF-1α 1:250, HIF-2α 1:500, IRF-1 1:500, VCAM-1 1:200). Membranes were washed with TBS–tween and then incubated with the appropriate HRP-conjugated secondary antibody for 1 h. After additional washing, immunogen levels were assayed with enhanced chemiluminescence (ECL reagent, GE Healthcare, Piscataway, NJ). Densitometry analysis was performed using GNU Image manipulation software (GIMP).

2.5. Quantification of mRNA and hnRNA levels

HDMEC were treated with DP or DMOG for 6 h followed by 4 h of 1000 U/mL TNFα. Total RNA was isolated with the Qiagen RNeasy Mini Kit (Valencia, CA) with inclusion of a DNase step using Qiagen RNase-free DNase. DNA-free RNA was then reverse transcribed with SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the random primer protocol. RT-PCR was performed with Sybr green chemistry (Power SYBR® Green PCR Master Mix, Applied Biosystems, Foster City, CA) on a 7500 Fast Real-Time PCR System (Applied Biosystems). The fold difference in mRNA expression relative to untreated cells was calculated employing the $\Delta\Delta C_T$ method with 18S as the endogenous control as described by the manufacturer. Real-time-PCR reactions were conducted in triplicate. All experiments were performed at least three times with the data from a representative experiment presented. The error in the calculation of fold difference was determined as described by the manufacturer. hnRNA levels were determined using primers which amplify intronic sequences as described previously [8]. Primer sequences are available upon request.

2.6. Immunocytochemistry

5A32s were grown on glass cover slips in 12 well plates. Cells were treated with DP or DMOG for 16 h followed by TNF-α at 1000 U/mL for 30 min. The cover slips were washed in ice-cold PBS and then cells were fixed with 3.7% paraformaldehyde for 7 min followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. The cover slips were washed again before blocking with 10% FBS in PBS. Next, they were incubated with anti-p65 antibody (1:300) for 30 min. The primary was removed with 3 washes of PBS and then Alexa Fluor 488 goat anti-rabbit (1:3000) (Invitrogen) was added for 30 min. After additional washing, the cover slips were mounted on glass slides using Prolong Gold mounting media (Invitrogen) and then fluorescence was visualized with a Leica DMR-E fluorescence microscope equipped with a Hammamatsu Orca camera.