Immunity, Vol. 2, 493–506, May, 1995, Copyright © 1995 by Cell Press

The Proteasome Pathway Is Required for Cytokine-Induced Endothelial-Leukocyte Adhesion Molecule Expression

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Summary

Multiple cell adhesion proteins are up-regulated in vascular endothelial cells in response to TNFα and other inflammatory cytokines. This increase in cell adhesion gene expression is thought to require the transcription factor NF-κB. Here, we show that peptide aldehyde inhibitors of the proteasome, a multicatalytic protease recently shown to be required for the activation of NF-κB, block TNFα induction of the leukocyte adhesion molecules E-selectin, VCAM-1, and ICAM-1. Striking functional consequences of this inhibition were observed in analyses of leukocyte-endothelial interactions under defined flow conditions. Lymphocyte attachment to TNFα-treated endothelial monolayers was totally blocked, while neutrophil attachment was partially reduced but transmigration was essentially prevented.

Introduction

The emigration of leukocytes from the bloodstream to extravascular sites involves a series of adhesive interactions with activated endothelium (reviewed by Springer, 1994; Carlos and Harlan, 1994). Initial random contact is followed by rolling of the leukocytes along the vessel wall. The selectin family of adhesion proteins and lectin counterreceptors mediate these initial adhesion events. In response to chemotactic factors and cytokines released in the local environment, leukocyte integrins are activated for subsequent interactions with endothelial ligands from the immunoglobulin gene superfamily. Leukocytes then flatten and spread on the endothelium in a process of adhesion strengthening. Tightly bound leukocytes then migrate to intercellular junctions, and diapedese between endothelial cells to enter the extravascular space and migrate to the area of the inflammatory or immune response. The definition of the molecules involved in these adhesive interactions has led to a working model for the molecular basis of leukocyte–endothelial interactions, and provided insights into therapeutic approaches to regulate the flammatory response.

Some of the adhesive proteins share a common regu tory feature in that endothelial expression is dramatica increased at sites of leukocyte recruitment. Endotheli leukocyte adhesion molecule-1 (E-selectin, ELAM-1, CD62E) is expressed in capillary and venular endotheli at sites of acute and chronic inflammatory responses (viewed by Pober and Cotran, 1990) and is rapidly induc by the tumor necrosis factor α (TNFα) and interleukir (IL-1) of the inflammatory cytokine, and bacterial lipopo saccharide (LPS) (Bevilacqua et al., 1989). The rise selectin expression may mediate the initial attachme and rolling of neutrophils (Lawrence and Springer, 19 1993; Abbassi et al., 1993). All selectins recognize sialat carbohydrate determinants on their counterreceptors (viewed by Lasky, 1992; Rosen, 1993). Vascular cell ad sion molecule-1 (VCAM-1, CD106), an immunoglobul like molecule, is expressed by a variety of vascular well as nonvascular cells. In endothelial cells, VCAM expression is induced by the inflammatory cytokin somewhat more slowly than E-selectin (Osborn et 1989). Endothelial expression of VCAM-1 mediates recruitment of lymphocytes, monocytes, and eosinop bearing α4β1 (VLA4, CD49d/CD29) or α4β7 integri (Elices et al., 1990; Chan et al., 1992) in chronic inflamn tory and immune responses (Cybulsky and Gimbro 1991; Briscoe et al., 1991; Freedman et al., 1990). Interc lular adhesion molecule-1 (ICAM-1, CD54), also immu globulin-like, interacts with leukocyte β2 integrins (CD1 CD18 and CD11b/CD18), and is important for transen thelial migration (Staunton et al., 1988; Smith et al., 198 Basal levels of ICAM-1 are present on unstimulated en thelial cells, but ICAM-1 also undergoes dramatic regulation in response to inflammatory cytokines (W heimer et al., 1992).

Cytokine induction of E-selectin, VCAM-1 and ICA genes leads to accumulation of high levels of mRN endothelial cells (Bevilacqua et al., 1989; Osborn et 1989; Staunton et al., 1988). Previous studies have d onstrated that the increase in these mRNAs after induct is due to an increase in the rate of gene transcript (Whelan et al., 1991; Neish et al., 1992; Wertheime al., 1992). To understand the mechanisms regulating cytokine-induced expression of these molecules, the man genes for E-selectin (Collins et al., 1991), VCA (Cybulsky et al., 1991), and ICAM-1 (Degitz et al., 19 Voraberger et al., 1991) have been cloned and the moter regions characterized. Cytokine responsivenes the E-selectin promoter requires multiple elements: th NF-κB binding motifs, a single ATF-2 binding site, multiple sites recognized by the high mobility group tein, HMGI(Y) (Whitley et al., 1994; Lewis et al., 19 Schindler and Baichwal, 1994). The organization of cytokine-inducible promoter is remarkably similar to virus-inducible promoter of the human interferon-β g

(Whitley et al., 1994). The VCAM-1 promoter contains two NF-κB sites, which are required for cytokine-induced transcriptional activation (Neish et al., 1992; Iademarco et al., 1992), as well as an interferon regulatory factor-1 (IRF-1) site, which cooperates with NF-κB to yield maximal cytokine responsiveness (Neish et al., 1995). Studies with the ICAM-1 promoter revealed that cytokine induction requires binding sites interacting with NF-κB and C/EBP (van de Stolpe et al.,1994; Hou et al., 1994). NF-κB may not only be required for these genes to respond to cytokine, but may also influence the temporal pattern of adhesive properties of endothelium during inflammatory responses.

NF-κB is a ubiquitous transcription factor and pleiotropic regulator of many genes involved in immune and inflammatory responses (reviewed by Grilli et al., 1993; Thanos and Maniatis, 1995). This family of dimeric transcription factor complexes consists of p50 (NF-kB1), and p52 (NF-kB2), which are both generated by proteolytic processing of precursor molecules, p105 and p100. The other members of this family, p65 (RelA), c-Rel, and RelB, have potent transactivation domains. p50/p65 is the predominant species found in endothelial cells, and binds to the NF-κB sites in both E-selectin and VCAM-1 (Read et al., 1994; Whitley et al., 1994; Neish et al., 1995). NF-κB is activated by posttranslational events involving phosphorylation and subsequent degradation of a cytoplasmic inhibitor, IκBα, which in resting cells prevents nuclear localization of NF-κB (reviewed by Beg and Baldwin, 1993). These factors interact in an autoregulatory mechanism: NF-κB mediates activation of the IκBα gene, resulting in replenishment of the cytoplasmic pool of its own inhibitor (Brown et al., 1993; Sun et al., 1993; Scott et al., 1993; de Martin et al., 1993).

The proteasome pathway has been linked to NF-κB activation. The proteasome is a 26S complex localized in the cytoplasm and nucleus, and contains a 20S proteolytic core (reviewed by Goldberg and Rock, 1992; Peters, 1994; Ciechanover, 1994). This nonlysosomal pathway of protein degradation is responsible for the turnover of abnormal and biologically active proteins in intact cells (reviewed by Ciechanover, 1994). Reagents that inhibit the proteasomal pathway have recently been used to demonstrate that the proteasome is required for processing and presentation of major histocompatibility complex (MHC) class I antigens (Rock et al., 1994). Processing of the p105 NF-κB precursor into the active p50 subunit also involves the proteasome proteolytic pathway. Inhibitors of the proteasome blocked IκBα degradation, and activation of NF-κB in response to TNFα in MG-63 and HeLa cells (Palombella et al., 1994). In endothelial cells, activation of NF-κB in response to TNFα occurs in parallel with degradation of IκBα (Read et al., 1994). In this study, we demonstrate that inhibitors that block the proteasome inhibit TNFα-induced cell surface expression of E-selectin, VCAM-1, and ICAM-1 in endothelial cells. These inhibitors exhibit profound functional effects by blocking static adherence of leukocytes, as well as attachment and transendothelial migration under defined flow conditions. We present biochemical and functional evidence that the mechanism of inhibition occurs at the level of NF-κB activation. These findings are consistent with the concept that the NF-κB/IκBα system may play a pivotal role in regulating leukocyte adhesion, and support the idea that rational manipulation of the level of specific transcription factors may regulate sets of genes controlling complex cellular events, such as those involved in endothelial pathophysiologic responses in the vasculature.

Results

Proteasomal Inhibitors Block TNFα-Induced Expression of Cell Surface Adhesion Molecules

To obtain evidence for involvement of the cellular proteasome in TNFα-induced expression of leukocyte adhesion molecules, the effects of a panel of proteasome inhibitors on surface expression of E-selectin were examined. The peptide aldehydes N-acetyl-leucinyl-leucinyl-norleucinal-H or Calpain inhibitor I (ALLN), N-carbobenzoxyl-leucinyl-leucinyl-norvalinal (MG115), and carbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG132) have been shown to inhibit the proteolytic activity of the 20S and 26S proteasomal complex (Rock et al., 1994). These three inhibitors also affect lysosomal proteases, including cathepsin B and calpain, with similar potency to a structurally related compound, N-acetyl-leucinyl-leucinyl-methional or Calpain inhibitor II (ALLM), a much weaker inhibitor of the proteasome (Rock et al., 1994). Whereas all four compounds have similar inhibitory activity against cathepsin B and calpain, their distinct inhibitory properties towards the proteasome makes them extremely useful reagents to distinguish between cellular pathways involving the proteasome or cathepsin/calpain-mediated protein degradation or processing.

Primary cultures of human umbilical vein endothelial cells (HUVECs) in microtiter plates were pretreated for 1 hr with the indicated concentrations of ALLM, ALLN, MG132, and MG115, then challenged with TNFα for 4 hr in the continued presence of the inhibitors (Figure 1A). Cell surface expression of E-selectin, as measured by fluorescence immunoassay, was essentially absent in unactivated HUVECs, and dramatically increased in response to TNFα. Control levels were unaffected by ALLM (Figure 1A), ALLN, MG115, and MG132 (data not shown). Cytokine-induced expression of E-selectin was profoundly inhibited in a dose-dependent manner by ALLN, MG115, and MG132, agents with known inhibitory activity against the proteasome. In contrast, ALLM failed to inhibit E-selectin expression in response to TNFα. The inhibitors MG132 and ALLM (Figure 1B), and MG115 and ALLN (data not shown), had no effect on surface levels of p96, a constitutively expressed endothelial surface molecule that is not regulated by TNFα (Bevilacqua et al., 1989). P96 is now known to be endoglin (M. Frosch, B. Seed, and T. C., unpublished data), a transforming growth factor β–binding protein (Gougos and Letarte, 1990; Cheifetz et al., 1992; Bellon et al., 1993).

The proteasomal inhibitors exhibited similar effects on TNFα induction of VCAM-1 and ICAM-1 surface expression (Figures 1C and 1D). In these experiments, HUVECs were pretreated with inhibitors as in Figure 1A, but chal-

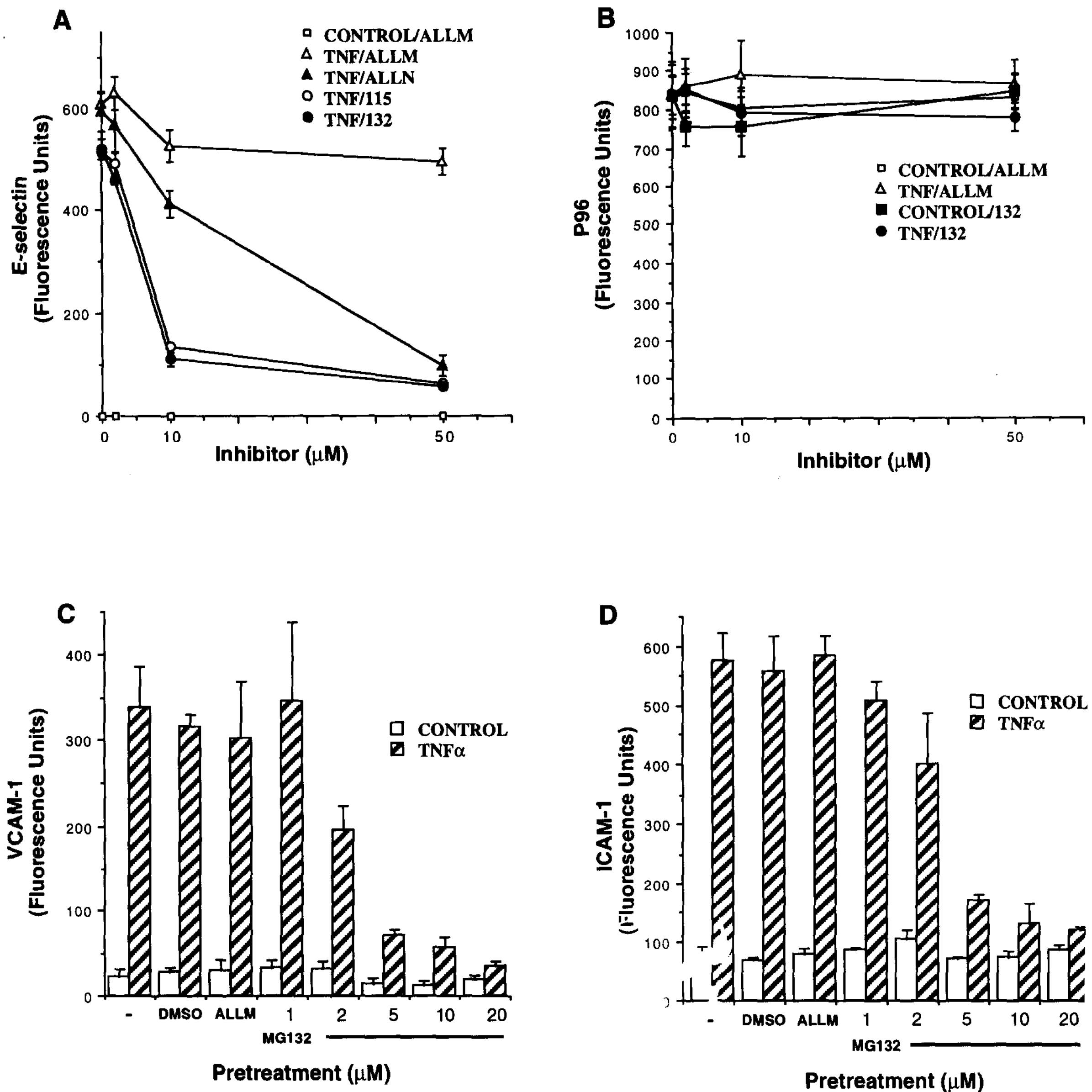

Figure 1. Inhibition of TNFα-Induced Cell Surface Expression of Leukocyte Adhesion Molecules by Proteasomal Inhibitors

HUVECs in microtiter plates were exposed to indicated concentrations of inhibitors for 1 hr, prior to the addition of 100 U/ml TNFα. Fluores immunoassays for E-selectin and p96 were performed at 4 hr ([A] and [B]), and VCAM-1 and ICAM-1 at 12 hr ([C] and [D]). Data are prese as mean ± SD, and are representative of three separate experiments.

lenged with TNFα for 12 hr, where maximal surface expression of these molecules occurs. MG132 completely blocked cytokine-induced VCAM-1 and ICAM-1 expression. During the time course of these experiments, basal ICAM-1 expression was not affected by proteasomal inhibitors. Similar results were observed with ALLN and MG115 (data not shown), but these were less effective than MG132. Again, the structural analog ALLM had no inhibitory activity. These findings establish that the proteasomal inhibitors decrease total expression of three leukocyte adhesion molecules.

To examine surface expression of these adhesion molecules on the entire population of HUVECs, flow cytometric analysis was performed on cells exposed to TNFα in absence and presence of 20 μM MG132. As showr Figure 2, the majority of HUVECs were positive E-selectin and VCAM-1 after 6 hr of TNFα treatment. S larly, ICAM-1 surface expression was significantly vated. In contrast, MG132 treatment prior to TNFα sig cantly reduced both the proportion of positive cells the mean fluorescence intensity of monoclonal antibo (MAbs) that recognize E-selectin, VCAM-1 and ICAI In general, MG132 reduced surface expression to ne that seen for control unactivated endothelial cells. MG did not alter adhesion molecule expression on control dia-treated HUVECs. As a further control, the surface

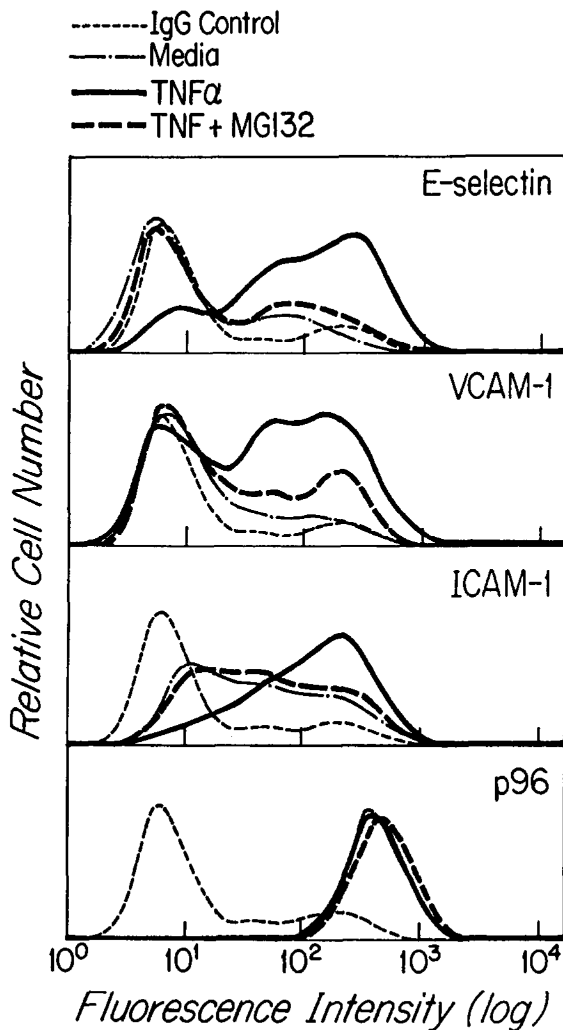

Figure 2. Endothelial Expression of E-selectin, VCAM-1, ICAM-1, and p96

Endothelial cells pretreated with control media, or TNFα (100 U/ml, 6 hr) without or with pretreatment with 20 μM MG132 for 1 hr were incubated with MAbs H4/18, E1/6, Hu5/3, and E1/1 to assess the surface expression of E-selectin, VCAM-1, ICAM-1, and p96, respectively, using indirect immunofluorescence flow cytometric analysis. The fluorescein-isothiocyanate intensity of endothelial cells stained with an isotype-matched nonbinding control (K16/6) IgG is indicated by a dotted line. Data presented are representative of three separate experiments.

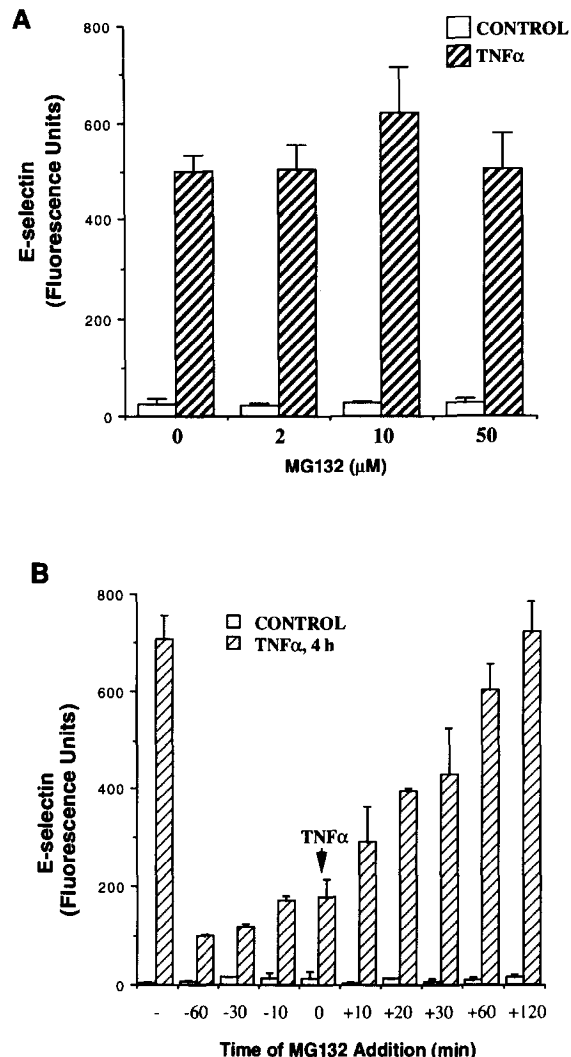

Figure 3. Effects of MG132 on HUVECs Are Reversible and Inhibition Is Time Dependent

(A) Reversibility of proteasomal inhibition and TNFα-induced E-selectin surface expression. HUVECs were exposed to the indicated concentrations of MG132 for 1 hr, washed twice in Hanks' balanced salt solution, and incubated in complete medium for 1 hr. Fresh medium with or without 100 U/ml TNFα was added, and fluorescent immunoassay for E-selectin performed at 4 hr.

(B) Effects of time of addition of MG132 on TNFα-induced E-selectin expression. MG132 (20 μM) was added at the indicated times relative to addition of 100 U/ml TNFα. Fluorescent immunoassay for E-selectin was performed after 4 hr of exposure to TNFα.

pression of p96 was not altered by MG132. These findings establish that proteasomal inhibitors decrease induced expression of all three leukocyte adhesion molecules in the entire population of endothelial cells.

Two conclusions can be made from these observations: first, the antiproteasomal agents block expression of the leukocyte adhesion molecules, as measured by both cell surface fluorescent immunoassay and flow cytometric analysis; and second, there is a direct correlation between the ability of the compounds tested to inhibit the proteolytic activity of the proteasome complex (Rock et al., 1994), and the ability of the inhibitors to block TNFα-induced expression of adhesion molecules. From this association, one can infer that these inhibitory effects on TNFα induction of adhesion molecule expression are mediated through the proteasome.

Effects of Proteasomal Inhibition on E-Selectin Expression Are Reversible and Time Dependent

To investigate whether the effects observed on TNFα-induced adhesion molecule expression in endothelial cells were reversible, HUVECs were treated with various concentrations of MG132 for 1 hr, rinsed, and allowed to recover for 1 hr before the addition of TNFα for 4 hr (Figure

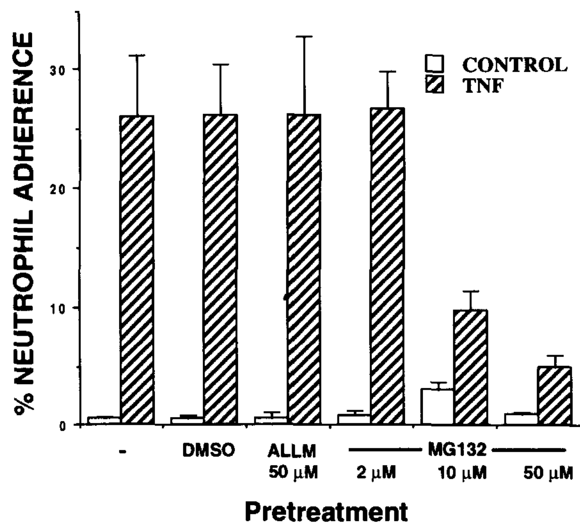

Figure 4. MG132, but Not ALLM, Inhibits TNFα-Induced Adherence of Neutrophils Under Static Conditions

HUVECs in microtiter plates were pretreated with the indicated concentrations of ALLM or MG132, or DMSO (0.05%) for 1 hr prior to addition of TNFα (100 U/ml) for 6 hr. Adherence of fluorescent-labeled peripheral blood neutrophils was determined as described in Experimental Procedures.

3A). In cells allowed to recover for 1 hr after inhibitor removal, TNFα-induced E-selectin expression was comparable with that seen in untreated cells at the concentrations tested (see Figure 1A). Thus, HUVECs fully recover their ability to respond to TNFα within 1 hr, indicating that the effects of MG132 are reversible. Similar results were observed in lymphoid cells, where the effect of proteasomal inhibitors on total protein turnover was reversible (Rock et al., 1994). The reversibility and lack of inhibition of p96 surface expression indicate that proteasomal inhibition is not toxic to the cells under the conditions employed.

To determine whether pretreatment with proteasomal inhibitors was necessary to block the effects of TNFα, the time of exposure to inhibitor was evaluated. MG132 was added to HUVECs at the indicated times before addition of TNFα for 4 hr, simultaneous with TNFα, or at the indicated times after addition of TNFα (Figure 3B). To inhibit TNFα-induced E-selectin expression, HUVECs must be exposed to MG132 either before or simultaneous with the addition of TNFα (Figure 3B). Thus, inhibition of the proteasome function interferes with early signaling events in response to TNFα.

Proteasomal Inhibitors Block TNFα-Induced Leukocyte Adherence, Rolling, and Transmigration

The localized adhesion of leukocytes to postcapillary and collecting venular endothelium occurs under flow conditions in vivo and is an essential process during the inflammatory response. Adhesion of peripheral blood neutrophils and lymphocytes to unstimulated endothelial cells is low. Cytokine activation of endothelium normally results in a surface that is hyperadhesive for nearly all classes of peripheral blood leukocytes. The functional cons quences of blocking endothelial adhesion molecule e pression by proteasomal inhibitors were evaluated by e amining adhesion of leukocytes to endothelium, both unc static conditions and under conditions of defined flow. A herence of peripheral blood neutrophils to HUVECs unc static conditions was enhanced by approximately 25-fc in response to 6 hr exposure to TNFα (Figure 4). Pretre ment of HUVECs with MG132 greatly reduced the level TNFα-enhanced neutrophil adherence, to less than 5-fc when compared with non-TNF-treated HUVECs. Simi results were obtained with U937 cells, a monocytic c line, where the optimal concentration of MG132 i blocking adherence under static conditions was 20 μ (data not shown).

To examine the effects of proteasomal inhibition ir system that mimics more closely conditions of flow in t microcirculation, the interactions between peripheral blc neutrophils and lymphocytes and TNFα-activated endot lium were examined under laminar flow (1.8 dynes/cm², e mated wall shear stress). By videomicroscopy, large nu bers of neutrophils attached, became firmly arrested, a transmigrated across TNFα-activated HUVEC mo layers, whereas few, if any, neutrophils attached to cont unactivated endothelial cells (Figure 5, first column, cc pare A with B). At higher magnification, many stably rested neutrophils flattened and spread on the apical s face, moved to intercellular borders, and migra beneath the activated endothelium. Transmigrated c are identified by arrows in Figure 5, first column, B anc The current findings are consistent with previous repc (Smith et al., 1989). Pretreatment with MG132 prior TNFα addition signficantly reduced neutrophil adhes (45% ± 7% inhibition) and inhibited ~90% of neutro transmigration (Table 1). Only a small amount of the re ual neutrophil adhesion in the presence of MG132 v blocked by an E-selectin MAb (data not shown). The id tity of the molecules mediating residual neutrophil ad sion are at present unclear.

Although neutrophil adhesion was reduced significan MG132 caused profound inhibition of transmigration. U closer inspection after several minutes of flow, m spread neutrophils were observed to project pseudop into the intercellular junctions, but were unable to pe trate fully between adjacent endothelial cells in orde migrate beneath the monolayers (Figure 5, second umn). For example, a neutrophil arrested on the ap endothelial surface has spread (Figure 5A, second umn, arrow) and then projected pseudopods into the in cellular borders between endothelial cells (Figure 5B, s ond column, pseudopods identified by small arro Failing to penetrate fully between the endothelial ju tions, this neutrophil migrated to a different region on same endothelial cell (Figure 5C, second column) anc peated the process of extending pseudopods into the dothelial borders (Figures 5D and 5E, second colu arrows identify pseudopods). Ultimately, this neutro failed to migrate beneath the endothelial monolayer seconds later was released from the apical surface (Fig 5F, second column) and was removed by flow. Re

Table 1. MG132 Blocks Leukocyte Adhesion and Transmigration Across TNFα-Activated HUVEC Monolayers under Flow at 1.8 dynes/cm²

	Neutrophils		Lymphocytes	
Treatments	Adhesion[a]	Transmigration[b]	Adhesion[a]	Transmigration[b]
Experiment 1				
Media	2 ± 2	0 ± 0	1 ± 1	0 ± 0
Media + 132	2 ± 2	0 ± 0	ND	ND
6 hr TNFα + ALLM	78 ± 4	36 ± 8	20 ± 10	2 ± 2
6 hr TNFα + MG132	43 ± 9	5 ± 4	1 ± 1	0 ± 0
Experiment 2				
Media	1 ± 1	0 ± 0	1 ± 2	0 ± 0
Media + 132	2 ± 1	0 ± 0	1 ± 2	0 ± 0
6 hr TNFα + ALLM	70 ± 10	31 ± 10	12 ± 4	3 ± 1
6 hr TNFα MG132	39 ± 13	4 ± 2	1 ± 2	0 ± 1

Leukocyte adhesion and transmigration were performed as described in Experimental Procedures. Data are mean ± SD.
[a] Adherent leukocytes per high powered field (40 × objective).
[b] Indicates transmigrated leukocytes per high powered (40 × objective).

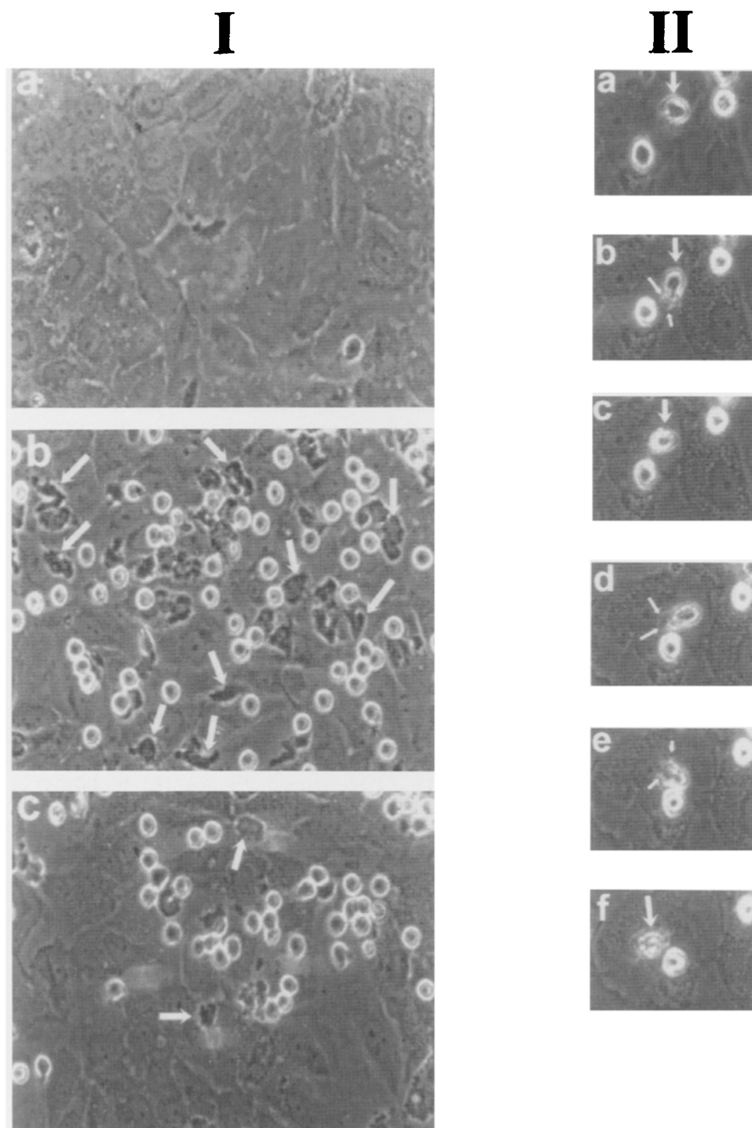

Figure 5. Neutrophil Rolling, Arrest, Spreading, and Transmigration Across TNFα-Activated Endothelium Under Laminar Flow Is Inhibited by MG132

(Column I) Various frames of video tape recordings of neutrophil adhesion under flow were digitized and photographed. Neutrophils were perfused across endothelial monolayers, which had been treated with control media (a), or 100 U/ml TNFα for 6 hr without (b) or with (c) pretreatment with 20 μM MG132 for 1 hr. Arrows indicate spread/transmigrated neutrophils.

(Column II) Sequential video frames depicting neutrophil arrest and spreading were digitized. The appropriate areas of interest from each digitized video frame were combined using commercial software to create the composite image (a–f). This composite image shows the various stages that occur between neutrophils and TNFα-activated HUVECs that have been pretreated with 20 μM MG132. (a) Arrow indicates neutrophil, which has arrested and is beginning to spread. (b) Same neutrophil (arrow) has projected pseudopods (small arrows) into the border between two endothelial cells. (c) Neutrophil (arrow) failed to migrate, moved to another area between two endothelial cells, and repeated the pseudopod extension (d and e, small arrows). (f) Neutrophil (arrow) retracts pseudopods, and moves to another area of the monolayer.

studies indicate that both adhesion molecules, including platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD-31) (Muller et al., 1993) and ICAM-1 (Smith et al., 1989), and endothelial-derived chemoattractant(s), including platelet-activating factor (Kuijpers et al., 1992) and IL-8 (Huber et al., 1991), are necessary for efficient neutrophil transmigration across cytokine-activated endothelium. The inability of neutrophils to transmigrate was not due to altered expression of PECAM-1 on HUVEC, as assessed by immunofluorescence microscopy (data not shown). Furthermore, although MG132 treatment prior to TNFα activation did reduce IL-8 production and secretion into culture supernatant, the differences were not significant ($p = 0.056$). Thus, inhibition of other steps in the migration pathway, including ICAM-1 expression, must be responsible for the decreased ability of the adherent neutrophils to migrate across endothelial monolayers under conditions of proteasomal inhibition.

Similar experiments with peripheral blood lymphocytes revealed that these cells also stably arrested, rolled, and transmigrated across TNFα-activated HUVEC, albeit in smaller numbers than neutrophils (Table 1). The inhibitory effects of MG132 were more dramatic for peripheral blood lymphocytes. Few freely flowing lymphocytes were able to attach to treated endothelial monolayers under flow, and thus, adhesion was reduced by >90% and transendothelial migration was absent. This result is consistent with recent findings that lymphocytes and neutrophils utilize distinct receptor–ligand pairs in the initial rolling phase of adhesion (Luscinskas et al., 1995; reviewed by Carlos and Harlan, 1994).

Effects of Proteasomal Inhibition on Induction of mRNA by TNFα

Activation of E-selectin, VCAM-1, and ICAM-1 gene expression is known to occur at the level of transcription (Whelan et al., 1991; Neish et al., 1992; Wertheimer et al., 1992). To examine whether inhibition of the proteasome blocked TNFα induction of steady-state transcript levels for E-selectin, VCAM-1, and ICAM-1, Northern blot analysis was performed (Figure 6A). HUVECs were incubated with or without MG132 for 1 hr, and stimulated with TNFα for 3 hr. In unstimulated HUVECs, endogenous message levels for E-selectin and VCAM are undetectable, and ICAM-1 is basally expressed at a low level (Figure 6A, lane 1). MG132 had no effects on basal levels of these transcripts (Figure 6A, lane 2). In the absence of inhibitor, 3 hr of TNFα treatment strongly induced message levels of all three molecules (Figure 6A, lane 3). In the presence of MG132, E-selectin transcript induction by TNFα was almost completely prevented, whereas transcript for VCAM-1 was totally blocked and ICAM levels remained at baseline (Figure 6A, lane 4). These results indicate that proteasomal inhibition may affect transcription of these genes. In contrast, MG132 pretreatment resulted in induction of IL-8 message (Figure 6A, lane 2), and TNFα-induced transcript levels for IL-8 were not inhibited by pretreatment with MG132 (lanes 3 and 4). These results are consistent with the lack of a significant effect of MG132 on IL-8 release into the media of TNFα-treated HUVECs (data not shown). IL-8 gene transcription has recently be shown to be under the cooperative control of C/EBPβ a NF-κB, possibly through p65 homodimers rather than h erodimeric p50/p65 (Kunsch and Rosen, 1993; Stein a Baldwin, 1993). Perhaps the strong cooperativity betwe this combination of transcription factors makes IL-8 tr scription less sensitive to effects of lower levels of nucl NF-κB, which are found in MG132-treated cells. No com nations of TNFα and MG132 affected the constitutive m sage for GAPDH, indicating that the levels of a const tively expressed gene were not affected by proteasor inhibitors (Figure 6A).

Proteasomal Inhibitors Reduce TNFα Activation of NF-κB in HUVECs

The E-selectin, VCAM-1, and ICAM-1 promoters con well-characterized NF-κB binding elements necessary transcriptional activation (Whelan et al., 1991; Neisl al., 1995; Voraberger et al., 1991). Earlier studies h shown that TNFα induces rapid nuclear localization of NF-κB subunits p50 and p65 in HUVECs (Read et 1994). Translocation of p50/p65 in HUVECs is accom nied by degradation of IκBα, which occurs by 15 r To examine the effects of proteasomal inhibitors on Iκ degradation, HUVECs were incubated with or without inhibitors for 1 hr prior to TNFα treatment for 15 min. level of IκBα was determined in cytosolic extracts by W ern blot analysis using an IκBα-specific antisera (Fig 6B). Both MG115 and MG132 markedly inhibited the of IκBα observed in response to TNFα (Figure 6B, c pare lanes 1 and 6 with lanes 12 and 13). In contr ALLN had a smaller effect on preventing IκBα degrada (Figure 6B, lanes 8 and 9), and ALLM did not prev degradation at all (lanes 10 and 11). For comparison, c solic levels of p50 and p65 remained constant in respo to TNFα and were not changed by exposure to the inl tors (Figure 6B). Cytosolic levels of p105 were not affe by TNFα or MG132, consistent with the long half-lif p105 when compared with IκBα (Donald et al., 1995). proteasomal inhibitors appeared to stabilize the phosp ylated form of IκBα, which was detectable only in extr from TNFα-treated cells in the presence of the inhibi (note the appearance of a doublet band, Figure 6B, la 8, 9, 12, and 13). Recent studies have shown that I phosphorylation is insufficient to cause dissociation f p65, and that subsequent degradation of IκBα is ne sary for nuclear translocation of NF-κB (Traenckner e 1994; Finco et al., 1994). Our results suggest that the teasome is involved in TNFα-induced degradation of I in HUVECs, and IκBα is phosphorylated in HUVECs p to degradation.

A time course of TNFα treatment in the presenc ALLM or MG132 was performed to examine the app ance of nuclear p50 and p65 (Figure 6C). In the prese of ALLM, TNFα induces nuclear translocation of p50 p65, which are maintained at a high level over time (Fi 6C, lanes 1–6), similar to what has been observe HUVECs treated with TNFα alone (Read et al., 19 However, in the presence of MG132, much less p50 p65 are translocated to the nucleus at 15 min, and tl

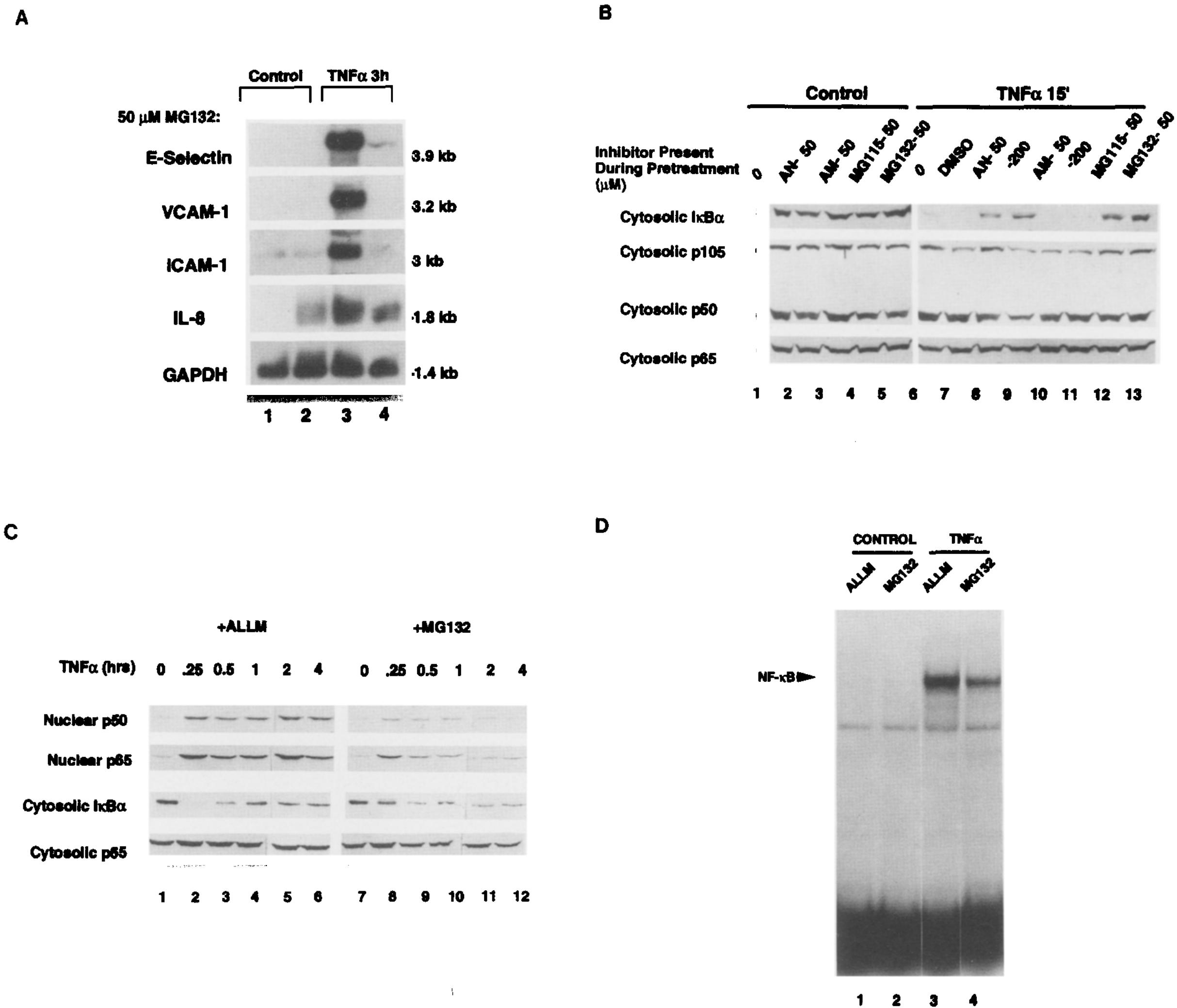

Figure 6. MG132 Blocks the Increase in Steady-State Transcripts Induced by TNFα and Inhibits TNFα-Induced Degradation of IκBα and Activation of NF-κB in HUVECs

(A) HUVECs were pretreated with 50 µM MG132 for 1 hr prior to the addition of TNFα (100 U/ml) for 3 hr. Total RNA was isolated and Northern blots prepared as described in Experimental Procedures. Transcript sizes are indicated on the right.

(B–D) HUVECs were pretreated with the indicated concentrations of inhibitors for 1 hr and exposed to TNFα (100 U/ml) for 15 min (B) or for the indicated times (C). Cytosolic and nuclear fractions were analyzed for IκBα, p65, and p105/p50 by Western blot (B and C). (D) Nuclear extracts from cells treated as described in (B) and exposed to TNFα for 30 min were analyzed for NF-κB DNA binding activity by EMSA using E-selectin PDI as a probe.

levels are not maintained, consistent with the lack of degradation of cytoplasmic IκBα (Figure 6C, lanes 7–12).

Results of the Western blots were supported by data obtained from electrophoretic mobility shift assays (EMSA) (Figure 6D), using the well-defined E-selectin PDI and PDIII NF-κB sites as probes. Treatment with MG132 prior to TNFα resulted in decreased PDI DNA binding activity from HUVEC nuclear extracts when compared with pretreatment with ALLM (Figure 6D, lanes 3 and 4). By supershift analyses, the composition of these protein–DNA complexes did not change in extracts from cells treated with TNFα alone, ALLM with TNFα, or MG132 with TNFα (data not shown). To quantitate the effects of MG132 on NF-κB binding, TNFα-induced DNA binding activity to E-selectin PDI and PDIII was analyzed by phosphoimage analysis and normalized to binding to an oligonucleotide containing a consensus YY1 binding site (Shi et al., 1991). Levels of YY1 protein and DNA binding in HUVECs are not affected by TNFα (M. Frosch and T. C., unpublished data). Comparison of quantitated binding to both PDI and PDIII shows that the degree of inhibtion by MG132 is greater than 50% (Table 2). Thus, although the proteasomal inhibitor MG132 does not completely block nuclear translocation of p50/p65 in HUVECs, the amount allowed into the nucleus is reduced. These diminished levels of NF-κB may not be sufficient to induce transcription of the NF-κB-dependent cell adhesion molecule genes.

TNFα-Induced Activation of NF-κB-Dependent Promoters Is blocked by MG132

To correlate the absence of adhesion molecule transcript and surface expression with diminished levels of nuclear

Table 2. Effects of Proteasomal Inhibition on NF-κB DNA Binding

Probe	PDI	PDIII	YY1	PDI/YY1	PDIII/YY1
Experiment 1					
ALLM (P.I. units/μl extract)	128 ± 18	69 ± 16	1200 ± 85	10.7	5.8
MG132 (P.I. units/μl extract)	53 ± 9	31.5 ± 10	1430 ± 112	3.7	2.2
Percent Inhibition by MG132				65.4%	62.1%
ALLM (P.I. units/μl extract)	248 ± 21	155 ± 14	2237 ± 112	11.1	6.9
MG132 (P.I. units/μl extract)	104 ± 8	64 ± 4	1919 ± 288	5.4	3.3
Percent inhibition by MG132				51.4%	52.2%

Data are mean ± SD from triplicate phosphoimage analysis.
MG132 inhibits TNFα-induced NF-κB DNA binding to E-selectin PDI and PDIII. Radiolabeled oligonucleotides (100,000 cpm) containing the or PDIII elements of the E-selectin promoter, or an oligonucleotide containing a consensus YY1 binding site were incubated with nuclear ext from TNFα-treated HUVECs pretreated with 20 μM ALLM or MG132 for 1 hr. Binding reactions were analyzed by EMSA as in Figure 6D, bound DNAs quantitated using a phosphoimager. Binding was analyzed relative to binding to an oligonucleotide containing a consensus site, to normalize the effective extract concentrations.

NF-κB, we tested drug effects on cytokine-inducible reporter genes. In these experiments, reporter constructs bearing NF-κB sites from VCAM-1 (both sites, Neish et al., 1995) and E-selectin (three copies of PDI, Whitley et al., 1994) were subcloned upstream of a constitutively active viral (SV-40) promoter and introduced into bovine aortic endothelial cells (BAECs). Transfected cells were then challenged with TNFα with or without 1 hr pretreatment with the inhibitory agents. As shown in Figure 7, a CAT reporter gene under control of the viral promoter exhibited a constitutive level of CAT activity that was not inducible with TNFα. This level of expression was not significantly affected by 2.5 or 5 μM ALLM or MG-132. In contrast, the same plasmids bearing NF-κB sites from VCAM-1 or E-selectin were inducible by TNFα. Strikingly, the TNFα induced activity of both was unaffected by 5 μM ALLM, but was totally abolished by pretreatment with 2.5 or 5 μM MG-132. Note that the level of activity of the viral reporter lacking the NF-κB sites was not significantly altered by exposure to either drug, demonstrating that these agents do not nonspecifically interfere with basal transcription. Reporter constructs driven by the intact core promoters of VCAM-1 and E-selectin were equally susceptible to inhibition of cytokine induction (data not shown). From these data, we conclude that the proteasomal inhibitor, MG-132, can abolish NF-κB-mediated TNFα inducibilty of VCAM-1 and E-selectin promoters. These studies con the functional significance of the decreased levels of clear NF-κB seen by Western blotting (see Figures 6B 6C) and gel shift analysis (see Figure 6D; Table 2), are consistent with the dramatic fall in cytokine-indu transcript levels seen by Northern blot analysis of F from inhibitor-treated endothelial cells (see Figure 6

Discussion

The proteasome is involved in both degradation of pro substrates, and in the limited proteolysis and proces that occurs during posttranslational modification of bio ically active proteins (reviewed by Ciechanover, 19 Our findings using a panel of peptide aldehydes with di ential inhibitory capabilities against the proteasome c onstrate that proteolytic activity of the proteasome i quired for TNFα induction of the leukocyte adhe molecules, E-selectin, VCAM-1, and ICAM-1. Effects o proteasomal inhibitor, MG132, on endothelial cells reversible, and inhibitor must be added prior to or sim neous with cytokine addition to be effective. Cyto induced transcript levels were almost abolished, inc ing interference with early signaling events. Biocher

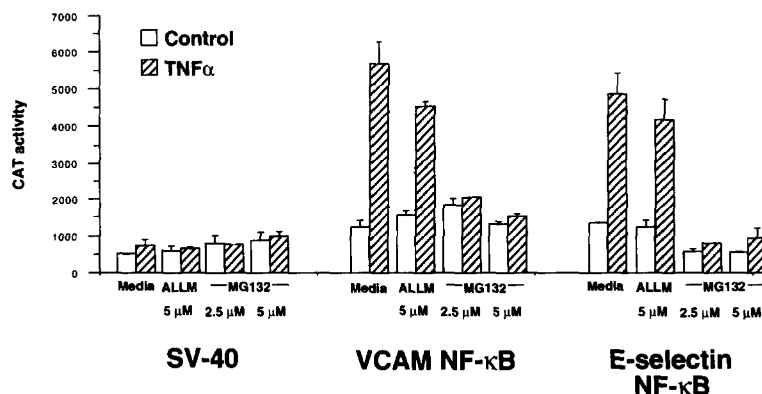

Figure 7. TNFα-Induced Activation of N Dependent Promoters Is Prevented by M
BAECs were transfected with 10 μg of S CAT, VCAM-NF-κB, or E-selectin NF-κ moter/reporter constructs. Following fection, cells were pretreated with the ind concentrations of ALLM or MG132 for 1 hr media with or without TNFα (200 U/ml) fo

studies revealed that TNFα-induced degradation of IκBα was blocked, and nuclear accumulation of NF-κB was reduced. TNFα inducibility of E-selectin and VCAM-1 NF-κB-dependent promoter–reporter constructs was abolished. Together, these results suggest that the decreased level of nuclear NF-κB that occurs in MG132-treated endothelial cells is insufficient to induce transcription of these NF-κB-dependent genes. The consequence of inhibiting expression of these leukocyte adhesion molecules was a dramatic inability of the endothelial cells to support leukocyte adhesion and transmigration.

These studies contribute to the list of physiological functions of the ubiquitin–proteasome proteolytic pathway (reviewed by Ciechanover, 1994; Goldberg and Rock, 1992; Peters, 1994). The degree of inhibition of induced leukocyte adhesion molecule expression by the peptide aldehydes correlated with the rank order of potency of these drugs to inhibit the proteolytic activity of the proteasome (Rock et al., 1994). This correlation suggests that induction of leukocyte adhesion molecules in endothelial cells occurs by the same inhibitor sensitive step. These results are similar to recent observations which demonstrated that the proteasome is involved in the degradation of long-lived proteins and is required for class I MHC antigen processing (Rock et al., 1994), as well as transcription factor activation (Palombella et al., 1994). The current study suggests the importance of the endothelial proteasome in the signal transduction events involved in adhesion molecule expression.

Interestingly, concentrations of MG132 that effectively blocked surface expression of E-selectin, VCAM-1, and ICAM-1, and IκBα degradation only partially prevented nuclear translocation of p50 and p65 in response to TNFα. Recent identification and cloning of another inhibitor of NF-κB, IκBβ, may provide further insight into these findings (Thompson et al., 1995). IκBα and IκBβ display distinct responses to activators of NF-κB. In contrast with IκBα, which is resynthesized following its degradation, IκBβ does not reappear under similar circumstances. The failure of IκBβ to be resynthesized suggests that unlike IκBα, IκBβ may not be up-regulated by NF-κB (reviewed by Thanos and Maniatis, 1995). These new findings, coupled with results of the current study, suggest that NF-κB activation in endothelial cells may be under control of an additional pathway, perhaps involving IκBβ.

The differences between the profound effects of MG132 on leukocyte adhesion molecule expression, and the partial inhibition of nuclear translocation of NF-κB, raises the possibility that signaling pathways in addition to NF-κB are being affected by proteasomal inhibitors. However, TNFα-induced activation of reporter constructs with NF-κB sites was abolished by pretreatment with MG132, while MG132 had no effect on the activity of a reporter lacking NF-κB sites. This apparent discrepancy between the partial inhibition of NF-κB activation and the complete blocking of adhesion molecule expression and reporter gene induction may reflect subtle control processes regulating expression of κB-dependent genes. Activation of NF-κB may be more complex and under additional levels of control besides nuclear translocation and DNA binding. A recent study has provided evidence that p65 is phosphorylated in response to TNFα in HeLa cells, and that DNA binding of p65 is phosphorylation dependent (Naumann and Scheidereit, 1994). Phosphorylation may have effects on other properties of p65, such as dimerization efficiency and transactivating potential. Alternatively, results of the current study may suggest that a threshold level of active NF-κB is required to induce transcription of the E-selectin, VCAM-1 and ICAM-1 genes. Multiple NF-κB sites with potentially different binding affinities exist in the transcriptional control regions of these genes. All three genes contain an array of binding sites recognized by other transcriptional activators. The decreased level of activated NF-κB in MG132-treated cells may result in a sharp decrease in transcription, which could lead to the results observed at the transcript and protein levels. This concept of a small change in the concentration of a transcriptional activator leading to a dramatic change in the level of gene expression has been well established (reviewed by Ptashne, 1992).

Using an in vitro flow chamber, we have established that inhibition of three TNFα-inducible endothelial leukocyte adhesion molecules markedly diminishes leukocyte arrest and transendothelial migration. Several features of our analysis of leukocyte interactions with endothelium treated with proteasomal inhibitors merit emphasis. First, the most dramatic affect was inhibition of peripheral blood lymphocyte initial attachment and rolling, and thus stable adhesion under flow. Recent studies have implicated P-selectin and other, as yet unidentified molecules, in $CD4^+$ T lymphocyte initial attachment and rolling on TNFα-activated endothelium under flow in vitro (Luscinskas et al., 1995). The current findings suggest these endothelial adhesion pathways are totally dependent on the NF-κB signaling mechanism. Second, E-selectin has been viewed as the major inducible endothelial adhesion molecule for neutrophil initial attachment and rolling on activated endothelium. Yet, despite the marked inhibition of E-selectin expression, many freely flowing neutrophils attached and rolled on MG132-treated endothelium. These data suggest that other inducible molecules, such as the L-selectin ligand (Spertini et al., 1991; Norgard-Sumnicht et al., 1993; Luscinskas et al., 1994) are either less susceptible to proteasomal inhibitors or that the signaling mechanisms for transcription are independent of the NF-κB system. Third, in the presence of MG132, most adherent neutrophils failed to diapedese across the endothelial monolayer. This may be due to the reduced levels of ICAM-1 expression or to a reduction in other factors, such as platelet-activating factor or IL-8, which have been implicated in neutrophil adhesion and diapedesis (Smith et al., 1989; Kuijpers et al., 1992; Huber et al., 1991). Fourth, the inhibition of neutrophil transmigration by MG132 raises the possibility that the endothelial proteasome may play a critical role in regulating leukocyte migration across endothelium and extravasation. Leukocyte binding may transmit a signal to endothelial cells that is mediated in part by the proteasome, which induces the endothelial cells to change shape and open intercellular junctions, thereby facilitating neutrophil passage across endothelium.

Normal function of the vasculature depends upon a series of carefully balanced and interrelated homeostatic mechanisms. These processes maintain the fluidity of the blood and the patency of vascular channels, while preventing loss of vascular contents. Pathologic changes can result in a variety of nonadaptive changes in the functional properties of the vasculature. These changes include alterations in the interactions of circulating cellular and macromolecular blood elements with the vessel wall. Such changes can result in hyperadhesiveness of leukocytes, increased cytokine and growth factor production, altered permeability of plasma lipoproteins, and alterations in the balance between procoagulant and fibrinolytic activities. Collectively, these changes have been designated "endothelial dysfunction" and this process plays an important role in the onset and progression of vascular disease (reviewed by Gimbrone, 1995). Several strategies have been proposed to decrease the physiologic consequences of endothelial dysfunction. For the most part, these are selective, in that they inhibit specific genes induced in dysfunctional endothelium (reviewed by Carlos and Harlan, 1994). For example, these strategies include blocking leukocyte–endothelial interactions with MAbs, soluble protein forms, or peptides derived from the adhesion molecules; blocking the cellular interactions with specific carbohydrate structures derived from the counterreceptors for the selectins; or decreasing induction of the adhesion proteins with specific antisense oligonucleotides. These strategies have been employed in animal models of ischemia/reperfusion injury, acute inflammation, and in models of transplant rejection. It has been noted that induction of many of the genes in dysfunctional endothelium occurs at the transcriptional level and that these genes share at least one regulatory element, a binding site for NF-κB (reviewed by Collins et al., 1993). There is evidence that this transcription factor is required for expression of some of these genes in endothelial cells. This suggests that general inhibitors of NF-κB activation may decrease the expression of a variety of genes associated with endothelial dysfunction and provide a broader novel approach to returning the endothelium to a quiescent state. Here, we demonstrate that treatment of endothelial cells with proteasomal inhibitors decreases nuclear accumulation of NF-κB by decreasing IκBα degradation and is correlated with decreased endothelial expression of three leukocyte adhesion molecules and decreased leukocyte transmigration. Therefore, the proteasomal pathway of NF-κB activation may represent a critical target for therapeutic intervention in the pathologic conditions associated with endothelial dysfunction.

Experimental Procedures

Cell Culture and Cytokine Treatment

HUVECs and BAECs were isolated and maintained in culture using previously described procedures (Gimbrone, 1976; Neish et al., 1992). For experiments on cytokine induction, cells were exposed to recombinant human TNFα (Genentech, San Francisco, California) at a final concentration of 100 or 200 U/ml in complete media. ALLN (Calpain inhibitor I) and ALLM (Calpain inhibitor II) were purchased from Boehringer Mannheim (Indianapolis, Indiana). MG132 and MG115 were provided by Myogenics, Incorporated (Cambridge, Massachusetts). Proteasomal inhibitors were prepared as 40 mM stock solution DMSO and added to complete medium before use.

For adhesion studies in the flow plate apparatus, HUVEC (pass 1–2) were plated at 80% confluence on fibronectin-coated (2 μg/ 25 mm circular glass coverslips (number 1 thickness, Thomas S tific, Swedesboro, New Jersey). For surface immunofluoresc studies, HUVEC were plated on 0.1% gelatin-coated or fibrone coated T-75 flasks (Corning). Experiments were initiated once monolayers reached confluence (typically after 48 hr).

Cell Surface Fluorescent Immunoassay

Cell surface binding assays were performed at 4°C on viable HU monolayers in microtiter plates, using saturating concentration MAb supernatants and fluorescent-conjugated $F(ab')_2$ goat anti-mu immunoglobulin G (IgG; Caltag Labs, San Francisco, California). bodies to E-selectin (H4/18), VCAM-1 (E1/6), ICAM-1 (Hu5/3), cl MHC (W6/32), and p96 (E1/1) were provided by Dr. M. A. Gimbr Jr. (Brigham and Women's Hospital, Boston). Fluorescent levels determined using an automated plate reader (Pandex, Baxter He care Corporation).

Leukocyte Isolation

Human neutrophils and lymphocytes were isolated from antico lated whole blood by centrifugation on Ficoll–Hypaque density g ent centrifugation at 15°C (LSM, Organon Teknika, Durham, N Carolina). The buoyant mononuclear cells were washed once and i bated in 150 cm^2 culture flasks at 37°C for 90 min to allow monoc to adhere firmly. Nonadherent lymphocytes (98% purity by Wri Giemsa stain) were washed once in RPMI, 10% fetal calf serum used in flow studies. Neutrophils were further purified by dextran mentation and hypotonic lysis as detailed previously (Luscinsk al., 1989), and were 95% pure by Wright–Giemsa stain.

Quantitative Static Adhesion Assay

Adhesion of unstimulated neutrophils or U937 cells to HUVEC m layers was determined as previously described (Westlin and brone, 1993). Following exposure to inhibitors, TNFα, or both, c ent HUVEC monolayers in microtiter plates were rinsed and coincul with bis-carboxyethyl-carboxyfluorescein acetoxymethly ester–la neutrophils or U937 cells (10^6/well) for 15 min at 37°C. Nonadh cells were removed by centrifugation. The number of adherent was quantified by lysing the contents of the wells in 10 mM Tris (pH 8.4), 0.1% SDS and fluorescence was determined in a micr plate fluorimeter. Percent adherence was calculated as follows percent of adherence = (lysate fluorescent units/total fluore units) × 100.

Endothelial–Leukocyte Interactions in a Parallel Plate Flow Chamber

The parallel plate flow chamber used for leukocyte adhesion defined laminar flow in this study has been described in detail (et al., 1992; Luscinskas et al., 1995). In brief, confluent endot monolayers on glass coverslips were incubated with culture r containing MG132 or ALLM (20 μM) for 1 hr and then treate media or media containing TNFα (100 U/ml; Genentech, Incorpor After 6 hr of incubation, HUVEC monolayers were placed in the ber and then lymphocytes or neutrophils (10^6 cells/ml) were through the chamber at 0.85 ml/min. The entire period of per was recorded on videotape. Leukocyte adhesion was determine 8–10 min of perfusion by analysis of 12–15 high power (40×) from videotape. Lymphocytes and neutrophils that had migrat neath the endothelial monolayer were easily distinguished b flattened and phase dense morphology under a 60× phase c objective (see Figure 6). In other analyses, selected video frame digitized and printed on a Tektronix II SDX digital printer using Photoshop software.

Immunofluorescence and Flow Cytometric Assays

Indirect immunofluorescence flow cytometric assays for E-se VCAM-1, ICAM-1 and p96 surface expression on control and treated HUVEC were performed as previously reported (Luscins al., 1989). The fluorescence of 10^4 cells was measured on a FA (Becton Dickinson) and the histograms are presented as mean c fluorescence (4 decade log scale) versus relative cell number.

Statistics
The adhesion data was collected using ANOVA and a Student's two sample t test was used to calculate statistical significance (Minitab statistical software, release 7, Minitab, Incorporated, State College, Pennsylvania). p values ≤ 0.05 were considered significant.

Northern Analysis
Total RNA from 10^7 cells was isolated by the guanidine isothiocyanate method (Sambrook et al., 1989). RNA was separated by electrophoresis on a 1% agarose formaldehyde gel, transferred to Hybond-N membrane (Amersham Corporation, Arlington Heights, Illinois) and immobilized by ultraviolet irradiation with an ultraviolet stratalinker 2400 (Stratagene, La Jolla, California). Blots were prehybridized for 6 hr and hybridized overnight at 42°C with ^{32}P-labeled probes (Megaprime kit, Amersham Corporation).

EMSA
Following experimental treatment of HUVECs, nuclear extracts were prepared as described previously (Whitley et al., 1994). Oligonucleotides were gel purified, annealed, and end-labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP (50 μCi at 3000 Ci/mmol, New England Nuclear, Boston, Massachusetts) and the Klenow fragment of Escherichia coli DNA polymerase I. Binding reactions and electrophoresis were performed as described (Whitley et al., 1994). Dried gels were scanned on a phosphoimager (Molecular Dynamics) and bound DNA was quantitated using ImageQuant software (Molecular Dynamics). The following oligonucleotides were utilized: E-selectin PDI, 5′-ggatGCCATT**GGGGATTTCC**TCTTTACTGGATGT-3′; E-selectin PDIII, 5′-aagcATCGTGGATAGGACA**GGGAAAGTTTT**TGGATC-3′; YY1 consensus, 5′cgctCC**GCGGCCATCTT**GGCGGCTGGT-3′. Lower case letters represent 5′ labeling overhang. Boldface letters indicate target sites.

Transfections and CAT Assays
BAECs were transfected with a modified calcium phosphate technique (Sambrook et al., 1989). Cells were transfected with 10 μg of reporter plasmid. Designated plates were treated with the indicated concentrations of MG132 or ALLN as described above for 1 hr. Following drug treatment, the media of appropriate plates were supplemented with TNFα (200 U/ml). Cells were harvested 8 hr after TNFα stimulation and CAT activity assayed as previously described (Whitley et at., 1994). Results are reported as the mean CAT activity of three independent experiments.

Western Blots and Antisera
Antisera to the Rel proteins p65 (N and C termini), and p105 were provided by N. Rice (National Cancer Institute, Frederick, Maryland; Rice and Ernst, 1993) and W. Greene (University of California, San Francisco, California; Sun et al., 1993). Antisera to IκBα was purchased from Santa Cruz Biotechnology, Incorporated (Santa Cruz, California). Following experimental treatment of HUVECs, cytosolic and nuclear extracts were prepared as described previously (Read et al., 1994; Whitley et al., 1994). Extracts were electrophoresed on 8% SDS polyacrylamide gels and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 5% methanol at 100V for 1 hr. Anti-p65, anti-p105, and anti-IκBα were used at 1:10,000, 1:10,000, and 1:1000 dilutions, respectively. Immunoreactive proteins were detected according to the enhanced chemiluminescent protocol (Amersham Corporation, Arlington Heights, Illinois) using 1:10,000 horseradish peroxidase-linked donkey anti-rabbit secondary antiserum. Blots were exposed to film for 1–5 min.

Acknowledgments

We thank Drs. M. A. Gimbrone, Jr. for MAbs, N. Rice and W. Greene for antisera to Rel proteins, M. Whitley for the E-selectin PDI reporter construct, W. Muller and R. McEver for antibodies to PCAM-1, and Myogenics, Incorporated for proteasomal inhibitors, MG132 and MG115. The expert technical assistance of K. Case, J. Kiely, A. Williams, W. Atkinson, and H. Ding is greatly appreciated. We are grateful to Dr. P. Tan for IL-8 assays. We thank Drs. J. Aster, A. Goldberg, K. Rock, and F. Stassen for helpful discussions. This work was supported by National Institutes of Health grants A12064 (to T. M.), HL03011-01 to (A. S. N.), HL47646 to (F. W. L.), and HL45462, HL35716, P0136028, and T32HL07627. T. C. is an Established Investigator of the American Heart Association.

Received December 15, 1994; revised February 28, 1995.

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