Journal of Cellular Biochemistry 91:926–937 (2004)

The Role of the Cytoskeleton in Cellular Adhesion Molecule Expression in Tumor Necrosis Factor-Stimulated Endothelial Cells

Elizabeth VandenBerg,[1] Maria D. Reid,[1] John D. Edwards,[1] and Harold W. Davis[1,2]*

[1]Department of Surgery, Division of Vascular Surgery, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, Ohio 45267-0558
[2]Department of Molecular and Cellular Physiology, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, Ohio 45267-0558

Abstract Leukocyte infiltration is a hallmark of the atherosclerotic lesion. These cells are captured by cellular adhesion molecules (CAMs), including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), platelet-endothelial cell adhesion molecule (PECAM), and E-selectin, on endothelial cells (EC). We examined the role of the actin cytoskeleton in tumor necrosis factor-alpha (TNF-α)-induced translocation of CAMs to the cell surface. Human aortic EC were grown on 96-well plates and an ELISA was used to assess surface expression of the CAMs. TNF-α increased VCAM-1, ICAM-1, and E-selectin by 4 h but had no affect on the expression of PECAM. A functioning actin cytoskeleton was important for VCAM-1 and ICAM-1 expression as both cytochalasin D, an actin filament disruptor, and jasplakinolide, an actin filament stabilizer, attenuated the expression of these CAMs. These compounds were ineffective in altering E-selectin surface expression. Myosin light chains are phosphorylated in response to TNF-α and this appears to be regulated by Rho kinase instead of myosin light chain kinase. However, the Rho kinase inhibitor, Y27632, had no affect on TNF-α-induced CAM expression. ML-7, a myosin light chain kinase inhibitor, had a modest inhibitory effect on the translocation of VCAM-1 but not on ICAM-1 or E-selectin. These data suggest that the surface expression of VCAM-1 and ICAM-1 is dependent on cycling of the actin cytoskeleton. Nevertheless, modulation of actin filaments via myosin light chain phosphorylation is not necessary. The regulation of E-selectin surface expression differs from that of the other CAMs. J. Cell. Biochem. 91: 926–937, 2004.

Key words: vascular cell adhesion molecule; intercellular adhesion molecule; E-selectin; tumor necrosis factor; endothelial cells; cytoskeleton

Among the earliest alterations in atherogenesis is the expression of cellular adhesion molecules (CAMs) on the surface of endothelial cells [van der Wal et al., 1992; Wood et al., 1993; Balaram et al., 1997]. These adhesion molecules are responsible for capturing circulating leukocytes and chaperoning them though the endothelial layer (intima) from the lumen of the blood vessel [Libby, 2002]. The major leukocyte involved in atherosclerosis is the monocyte and once it enters the subendothelial space it is transformed into a macrophage and can accumulate lipid [Ross, 1999]. The adhesion molecules include the P- and E-selectins, which are responsible for the initial capture of the leukocyte, vascular cell adhesion molecule (VCAM), and intercellular adhesion molecule (ICAM), which bind the leukocyte and allow it to roll along the endothelium and platelet endothelial adhesion molecule (PECAM), which leads the leukocyte through the endothelial monolayer, a process called diapedesis [Libby, 2002]. Adhesion molecule transcription requires the activation, and translocation to the nucleus, of nuclear factor-κB [NF-κB; Collins et al., 1995; Kim et al., 2001]. However, with the exception of P-selectin [Blagoveshchenskaya

Grant sponsor: American Heart Association (Ohio Valley Affiliate to HWD).

*Correspondence to: Harold W. Davis, University of Cincinnati Medical Center, 231 Albert Sabin Way, P.O. Box 670558, Cincinnati, OH 45267-0558.
E-mail: harold.davis@uc.edu.

Received 16 October 2003; Accepted 21 November 2003
DOI 10.1002/jcb.20011

et al., 2002], the signaling mechanisms involved in adhesion molecule migration to the cell surface are largely unknown.

Tumor necrosis factor-alpha (TNF-α), one of the major proinflammatory cytokines associated with atherogenesis, exerts some of its effects by inducing expression of adhesion molecule on endothelial cells and by increasing endothelial migration and the permeability of the endothelial monolayer [Deli et al., 1995; Madge and Pober, 2001; Gao et al., 2002]. The latter two phenomena are known to be regulated by the actin cytoskeleton and are dependent on phosphorylation of myosin light chains [Gotlieb et al., 1987; Lampugnani et al., 1990; Garcia et al., 1995; Dudek and Garcia, 2001]. Myosin light chain phosphorylation is regulated by two enzymes, myosin light chain kinase, which directly phosphorylates the light chains [Adelstein et al., 1981; Garcia et al., 1995], and Rho kinase, which can either directly phosphorylate the light chains [Amano et al., 1996] or phosphorylate myosin phosphatase to inhibit its dephosphorylating action, thus, indirectly increasing myosin light chain phosphorylation [Kimura et al., 1996]. Phosphorylation of myosin light chains regulates the formation of actin stress fibers [Goeckeler and Wysolmerski, 1995; Zhao and Davis, 1999], which are involved in cell contraction and integrin-mediated cell adhesion [Gotlieb et al., 1987; Lampugnani et al., 1990; Garcia et al., 1995; Dudek and Garcia, 2001; Miranti and Brugge, 2002]. Importantly, myosin light chain phosphorylation is required for migration of leukocytes across the endothelial monolayer [Garcia et al., 1998; Saito et al., 1998, 2002]. In these studies, we investigated the role of actin reorganization in TNF-α-induced adhesion molecule expression on the surface of human aorta endothelial cells.

MATERIALS AND METHODS

Human aortic endothelial cells (HAEC) were obtained from BioWhittaker (Walkersville, MD) and grown in EGM-2 media (also from BioWhittaker). TNF-α was purchased from PeproTech (Rocky Hill, NJ). Cytochalasin D, jasplakinolide, and Y27632 were from Calbiochem (LaJolla, CA). Ethylenediamine tetraacetic acid (EDTA), *O*-phenylenediamine, 30% hydrogen peroxide, ML-7, and the E-selectin antibody were obtained from Sigma Chemical Co. (St. Louis, MO). The antibody against VCAM-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the antibodies against ICAM-1 and PECAM were obtained from R&D Systems (Minneapolis, MN). The diphosphorylated myosin light chain antibody was purchased from Cell Signaling Technologies (Beverly, MA) and the total myosin light chain antibody was kindly provided by Dr. C.S. Packer (Indiana University School of Medicine). Secondary antibodies conjugated to horseradish peroxidase were from Bio-Rad (Hercules, CA) and the secondary antibody conjugated to Texas Red, as well as the BODIPY FL Phallacidin were from Molecular Probes (Eugene, OR). Vectashield mounting media containing DAPI for fluorescent microscopy was obtained from Vector Laboratories, Inc. (Burlingame, CA).

HAEC grown to confluence in 96-well plates coated with 0.2% gelatin, were treated with 20 ng/ml TNF-α for various periods of time. Inhibitors were added 30 min prior to the addition of the TNF-α. To terminate the experiment, the media was quickly removed and the cells were washed with PBS (all volumes for this assay were 100 μl, unless otherwise stated) and then fixed with 2% paraformaldehyde for 10 min at room temperature. The cells were washed and incubated with 5% BSA in PBS for 10 min at room temperature then incubated with primary antibodies (prepared in 1% BSA in PBS) against VCAM-1 (10 μg/ml), ICAM-1 (2.5 μg/ml), PECAM (2.5 μg/ml), or E-selectin (50 μg/ml) for 60 min at 37°C. The cells were washed three times with PBS and anti-mouse IgG conjugated to horseradish peroxidase (10 μg/ml) in 1% BSA was added and the cells were incubated for 45 min at 37°C. The cells were washed four times with PBS and *O*-phenylenediamine (0.4 mg/ml; with 0.4 μl/ml 30% hydrogen peroxide added immediately before) was added. When color developed (approximately 4 min) the reaction was terminated with 50 μl of sulfuric acid. The plates were read at 490 nm.

To examine phosphorylation of myosin light chains, the cells were grown to confluence in 6-well plates and following treatment, were washed with ice-cold PBS and harvested in 0.2 ml of extraction buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, and 1% Igepal CA-630). After cell debris was spun down, equal quantities of protein were separated by 15% SDS–PAGE and transferred to PVDF, detected by immunoblot-

ting and quantified by densitometry. Total cellular protein concentrations were measured with the Pierce (Rockford, IL) bicinchoninic acid protein assay. Total adhesion molecule expression was assessed by separating the proteins on non-denaturing SDS–PAGE and blotting as described above.

For some experiments, the cells were grown on glass coverslips and prepared for fluorescent or confocal microscopy as we have previously described [Neltner et al., 2000]. The fluorescently labeled cells were viewed with either an epifluorescent Ziess Axioplan microscope or a Ziess LSM-510 confocal laser-scanning microscope. Objectives (64×) were used on both.

A lactate dehydrogenase assay kit (Sigma Chemical Co.) was used to assess cell death. None of the reagents increased cell death. All data shown are from at least three experiments conducted in duplicate and statistical significance was determined by ANOVA followed by Tukey's test.

RESULTS

Time Course

Dose response experiments indicated that 20 ng/ml TNF-α provided a maximal surface expression of the CAMs (data not shown). This concentration of TNF-α led to increases in surface expression of VCAM-1, ICAM-1, and E-selectin but did not alter PECAM expression at any time point examined (Fig. 1). Since PECAM expression was unchanged in response to TNF-α, we did not investigate it further. The surface expression of the other three adhesion molecules was maximally elevated by 4 h and remained elevated for at least 24 h if TNF-α remained present. All subsequent experiments were conducted with 4 h of TNF-α treatment. These data are consistent with those reported previously [Hahne et al., 1993].

Confocal Microscopy of Endothelial Monolayers

To ensure that the adhesion molecules that we were detecting by the ELISA were on the cell surface and not the total CAM expression, we examined the cells with confocal microscopy. The cells were treated exactly as for the ELISAs except that the secondary antibody was conjugated to Texas Red instead of horseradish peroxidase. Twenty five slices were recorded (~0.3 μm thickness) for each sample and a Z stack was created to show a side view of the cells. As revealed in Figure 2, all of the staining for the CAMs is on the cell surface, mainly on the apical membrane. When the HAEC are permeabilized with Triton X-100 CAMs are visible in the cytosol, indicating that with the ELISAs we

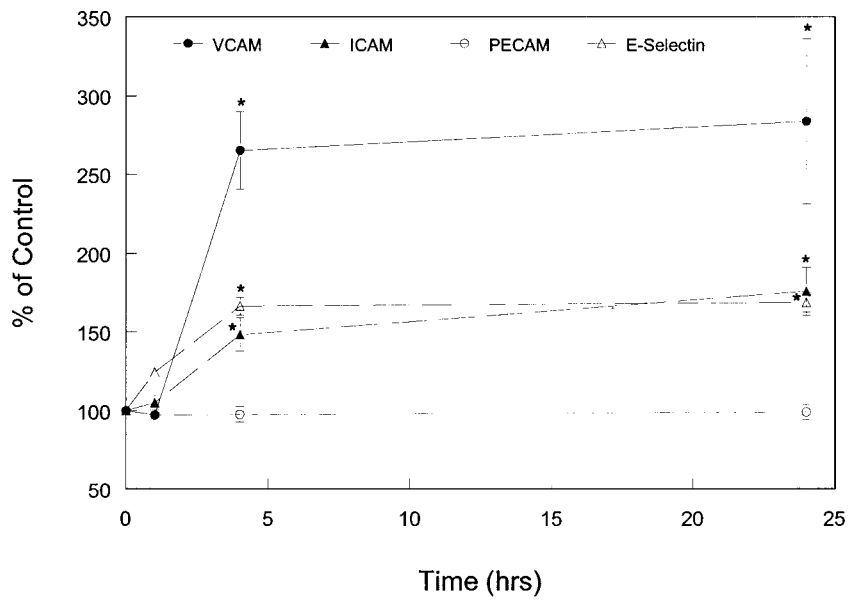

Fig. 1. Time course of adhesion molecule expression on the surface of human aortic endothelial cells (HAEC). HAEC were treated with 20 ng/ml tumor necrosis factor-alpha (TNF-α) for the indicated times. The reactions were terminated and adhesion molecule expression was determined as described in Materials and Methods. $^*P < 0.05$, compared to control (no TNF-α).

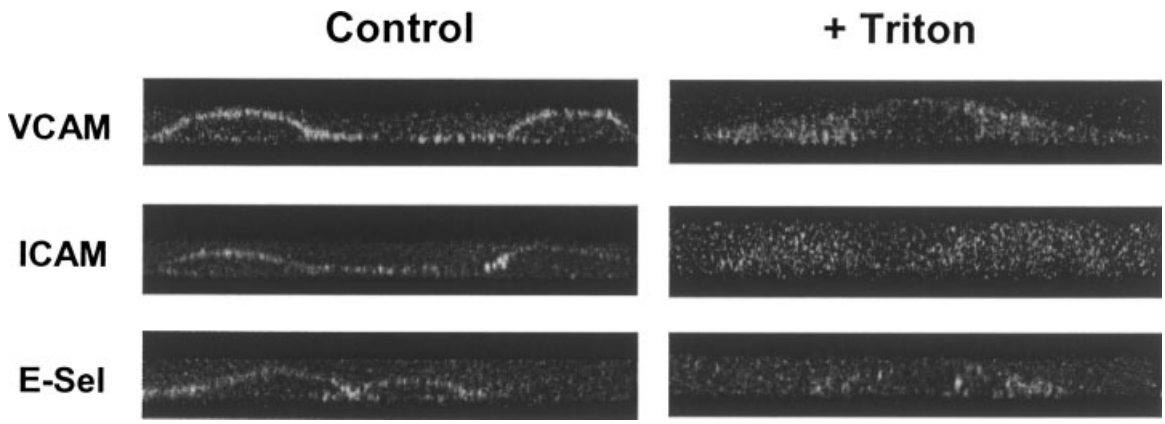

Fig. 2. Z slice of HAECs stained with antibodies to different adhesion molecules. The cells were treated with 20 ng/ml TNF-α for 4 h then processed for confocal microscopy as described in Materials and Methods. The antibodies stain vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and E-selectin primarily on the apical surface of the HAEC. Processing of the cells does not allow antibody entrance into the cytosol to stain intracellular CAMs (**left panels**). However, when the cells are permeabilized with 0.2% Triton X-100, the CAMs are visible in the cytosol (**right panels**).

are detecting only surface and not total CAM expression. Without TNF-α treatment, the amount of staining on the cell surface was barely detectable for ICAM-1 and E-selectin and undetectable for VCAM-1 (data not shown).

Filamentous Actin Disruption

The actin cytoskeleton is involved in integrin signaling and is rearranged when leukocytes bind to endothelial cells. We, therefore, examined the effect of TNF-α on F-actin distribution (Fig. 3). As shown, stress fibers running across the cells are present in untreated cells but their number and thickness is increased by TNF-α treatment. There is also an increase in the total amount of F-actin in treated cells. In addition, TNF-α causes the cells to pull apart from each other leaving holes in the monolayer. By either disrupting the actin filaments with cytochalasin D or rigidly stabilizing them with jasplakinolide we were able to attenuate the TNF-α-induced translocation of VCAM-1 and ICAM-1 to the

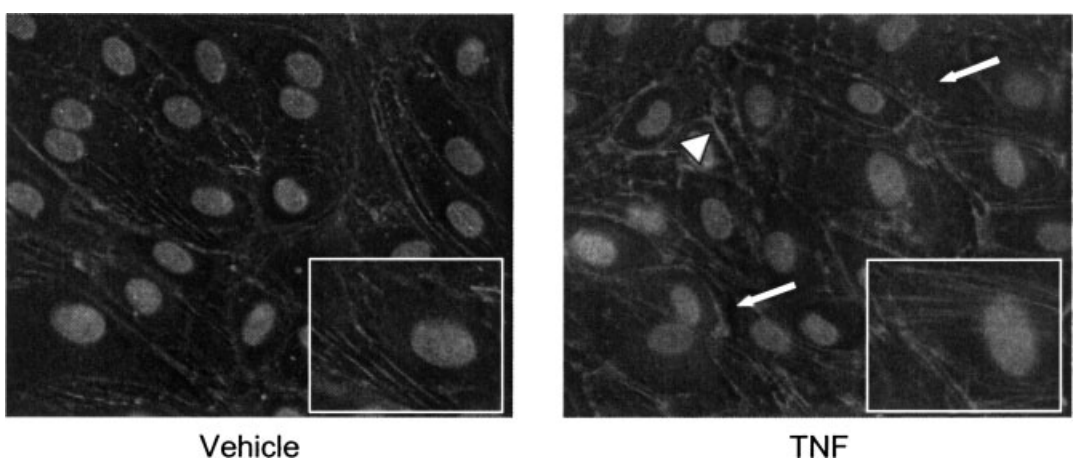

Fig. 3. The effect of TNF-α on filamentous actin arrangement in HAEC. HAEC were treated with 20 ng/ml TNF-α for 4 h then fixed and permeabilized. Actin was stained with BODIPY FL Phallacidin and the nuclei were stained with DAPI. The monolayers were examined with an epifluorescent microscope. TNF-α treatment induced the formation of total F-actin (phallacidin only binds F-actin, not G-actin) and more stress fibers with thicker bundles of actin filaments (arrowheads). TNF-α treatment also resulted in holes in the monolayer (arrow). The micrographs are at 63× magnification. The inserts are at 252×.

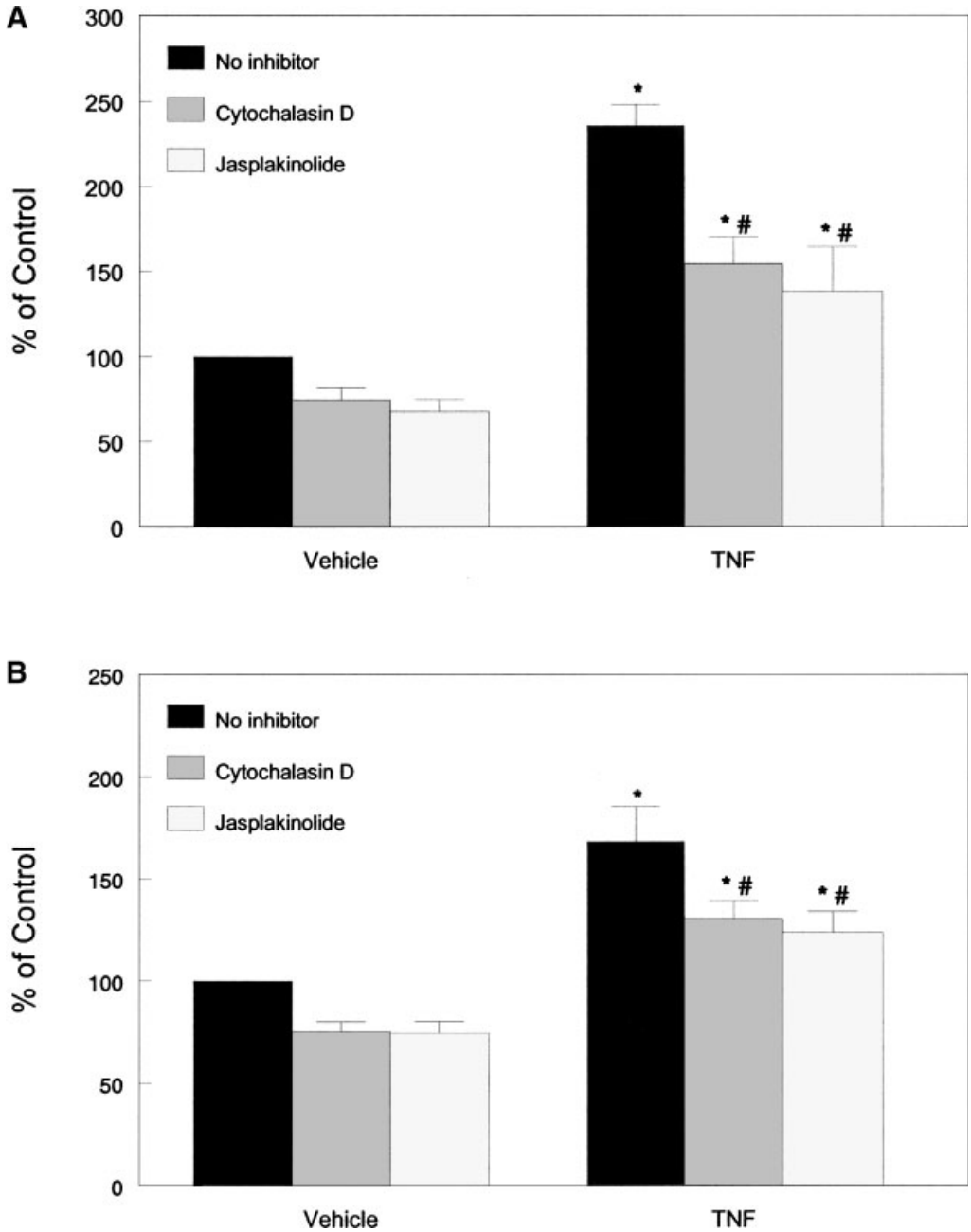

Fig. 4. The effect of cytoskeletal disruption on adhesion molecule expression. HAEC were stimulated with 20 ng/ml TNF-α for 4 h and adhesion molecule expression was determined as in Materials and Methods. Cytochalasin D (5 μM) or 100 nM jasplakinolide was added 30 min prior to the TNF-α (**A**) VCAM-1, (**B**) ICAM-1, and (**C**) E-selectin. *, $P < 0.05$, compared to control (no TNF-α) for each inhibitor. #, $P < 0.05$, compared to TNF-α treated, no inhibitor.

HAEC surface (Fig. 4A,B). These compounds had little or no affect on E-selectin surface expression (Fig. 4C). As shown in Figure 5, neither cytochalasin D nor jasplakinolide alters basal or TNF-induced whole cell expression of ICAM or E-selectin. However, cytochalasin D, but not jasplakinolide, does interfere with TNF-induced synthesis of VCAM.

Myosin Light Chain Kinase and Rho Kinase

As mentioned above, the phosphorylation of myosin light chains is required for filamentous

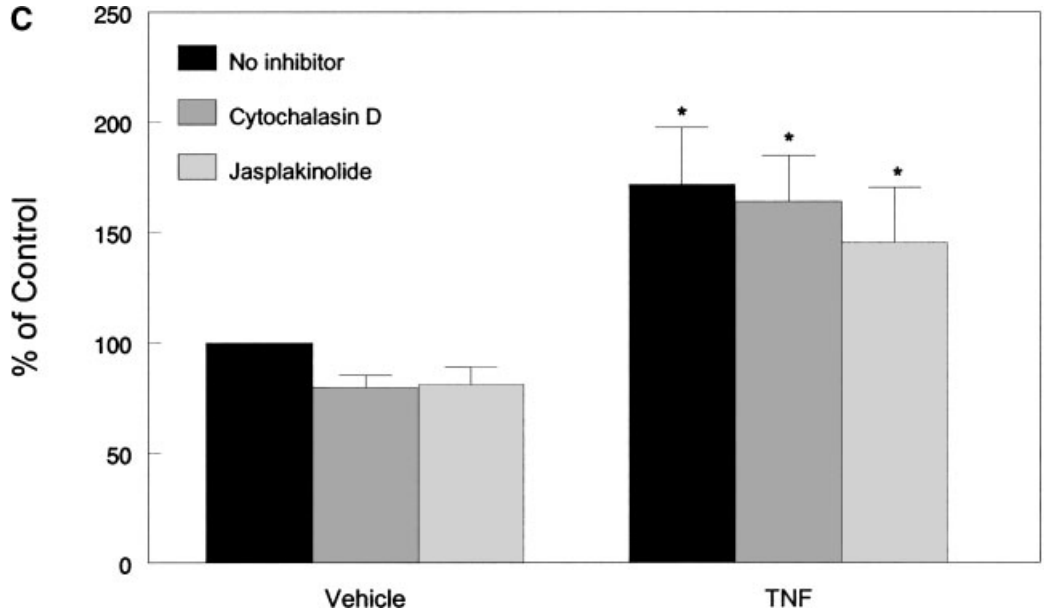

Fig. 4. (*Continued*)

actin stress fiber formation. If these stress fibers were necessary for adhesion molecule translocation to the cell surface, inhibition of myosin light chain phosphorylation would inhibit this process. TNF-α stimulated diphosphorylation of myosin light chains in a biphasic manner; the phosphorylation peaked at 1 and 4 h (Fig. 6). This phosphorylation was unaffected by ML-7, a myosin light chain kinase inhibitor, but was abolished by the Rho kinase inhibitor, Y27632 (Fig. 7). Basal myosin light chain phosphorylation also appeared to be dependent on Rho kinase activity.

Y27632, while completely inhibiting myosin light chain phosphorylation, had no affect on the expression of any of the CAMs (Fig. 8). Conversely, ML-7, modestly inhibited the TNF-α-induced surface expression of VCAM-1 but not of ICAM-1 or E-selectin (Fig. 9). These data suggest that myosin light chain phosphorylation is not critical for conveyance of adhesion molecules to the cell surface.

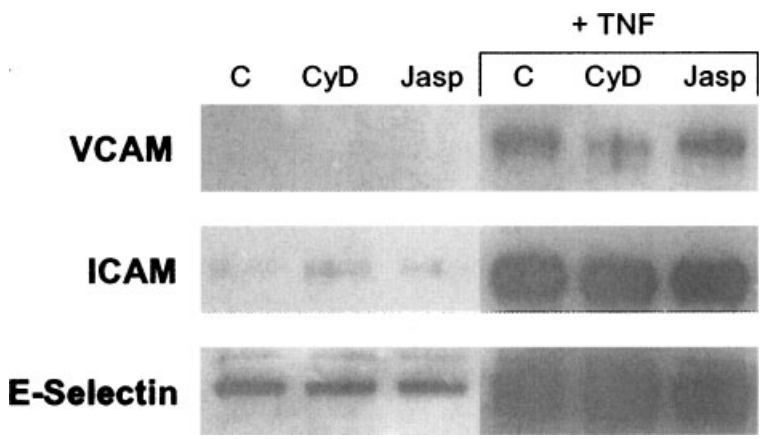

Fig. 5. The effects of TNF-α and cytoskeletal disruption on whole cell expression of adhesion molecules. HAEC were treated for 4 h with vehicle or 20 ng/ml TNF-α −/+ cytoskeletal disruptors as in Figure 4. Following the incubations the proteins were separated by non-denaturing SDS–PAGE and blotted as described in Materials and Methods. C, control (no inhibitor); CyD, cytochalasin D (5 μM); Jasp, jasplakinolide (100 nM).

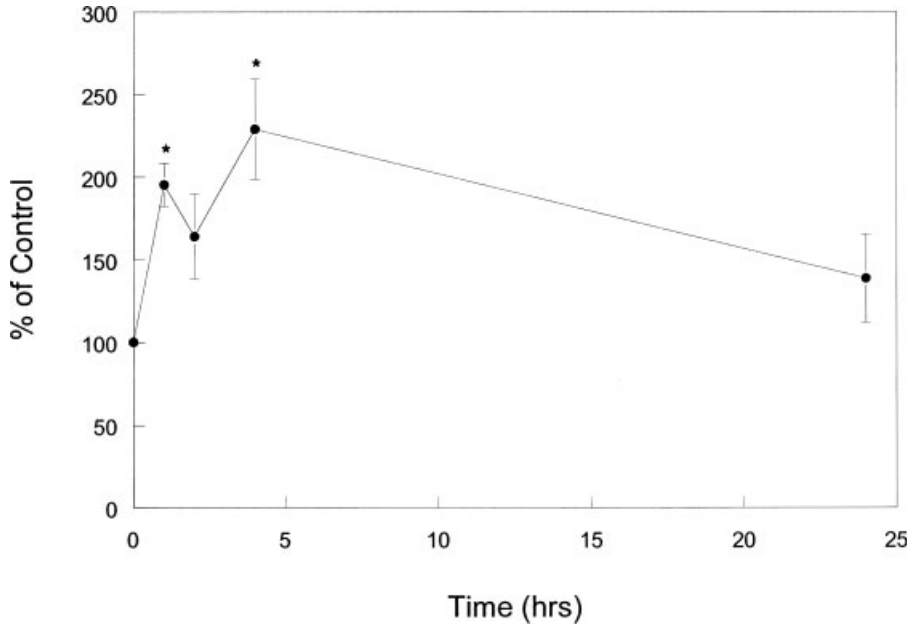

Fig. 6. Time course of myosin light chain phosphorylation in response to TNF-α. HAEC were treated with 20 ng/ml TNF-α for 0–24 h. The reactions were stopped and processed for SDS–PAGE as described in Materials and Methods. The PVDF membrane was probed with a diphosphorylated myosin light chain antibody then the membrane was stripped and reprobed with an antibody that recognizes total myosin light chains. Shown is the ratio of diphosphorylated myosin light chain to total myosin light chain. *, $P < 0.05$, compared to control (no TNF-α).

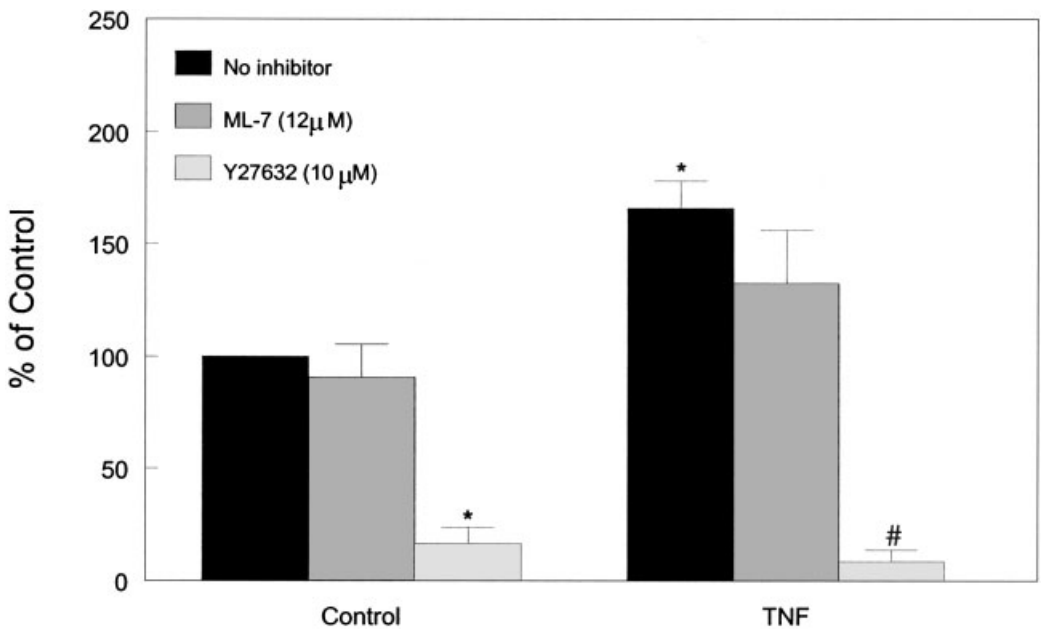

Fig. 7. Effect of ML-7 and Y27632 on TNF-α-induced myosin light chain phosphorylation. HAEC were treated for 4 h with 20 ng/ml TNF-α then processed as described in Figure 6. Some cells were treated with 12 μM ML-7 or 10 μM Y27632 for 30 min before the addition of TNF-α. *, $P < 0.05$, compared to control (no TNF-α) for each inhibitor. #, $P < 0.05$, compared to TNF-α treated, no inhibitor.

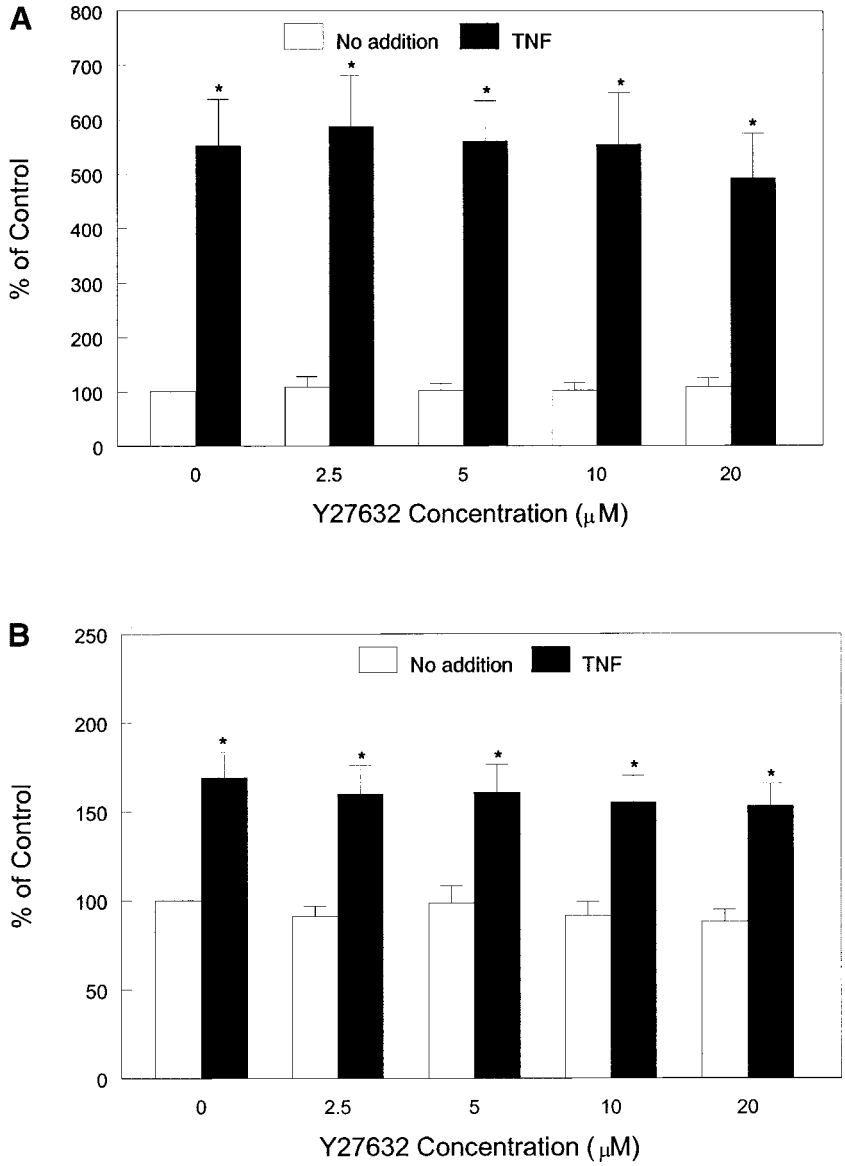

Fig. 8. The effect of Y27632 on cell surface adhesion molecule expression. HAEC were incubated with various concentrations of Y27632 for 30 min then stimulated for 4 h with 20 ng/ml TNF-α. VCAM-1 (**A**), ICAM-1 (**B**), and E-selectin (**C**) were detected by ELISA as described in Materials and Methods. Inhibition of Rho kinase with Y27632 had no affect on the expression of any of the CAMs. *, $P < 0.05$, compared to control (no TNF-α) for each inhibitor. #, $P < 0.05$, compared to TNF-α treated, no inhibitor.

DISCUSSION

Capture and uptake of leukocytes, mainly monocytes and T-lymphocytes, is a critical early event in the development of atherosclerosis. The capture of leukocytes is dependent on the expression of CAMs on the endothelial cell surface. Transcription of the adhesion molecules requires NF-κB activation. But the signaling pathways involved in the translocation of these proteins from the cytosol to the cell membrane have received little attention. Several groups have reported that protein kinase C or tyrosine kinases are important in adhesion molecule translocation [Lane et al., 1990; Deisher et al., 1993; Krunkosky et al., 1996; May et al., 1996] but, to our knowledge, the role of the cytoskeleton has not been investigated. When leukocytes bind to endothelial cells, an actin-dependent-adhesion molecule clustering occurs

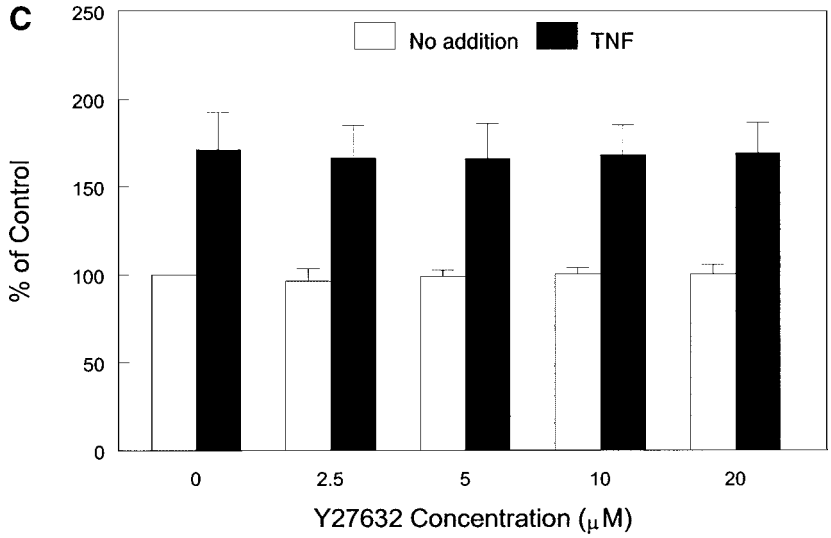

Fig. 8. (*Continued*)

[Wojciak-Stothard et al., 1999; Matheny et al., 2000]. This clustering is dependent on myosin light chain phosphorylation [Garcia et al., 1998; Saito et al., 1998, 2002] but appears to be due to Rho kinase activation and not myosin light chain kinase [Saito et al., 2002]. Because of the critical role of the actin cytoskeleton in adhesion molecule clustering and in integrin function [Lampugnani et al., 1990; Miranti and Brugge, 2002], we disrupted the actin cytoskeleton to determine whether this would influence adhesion molecule expression.

Actin is present in cells in two forms: globular (G)-actin, which are single subunits of actin molecules and filamentous (F)-actin, which are polymers of G-actin. The latter is what the stress fibers are composed of. Cytochalasin D, which inhibits actin polymerization, attenuated the TNF-α-induced surface expression of VCAM-1 and ICAM-1. However, it had no effect on E-selectin expression. Similar data were obtained with jasplakinolide, which rigidly stabilizes the actin filaments. These data suggest that translocation of VCAM-1 and ICAM-1 requires cycling between G-actin and F-actin. E-selectin translocation is independent (or at least less dependent) of the actin cytoskeleton. TNF considerably increases the synthesis of all of these CAMs (Fig. 5). Importantly, however, the synthesis of the CAMs is not affected by jasplakinolide and only VCAM expression is attenuated by cytochalasin D. This suggests that with the exception of VCAM, the cytoskeleton does not regulate CAM synthesis. VCAM synthesis appears to require an intact cytoskeleton but not the actin filament cycling that is necessary for translocation of this protein to the cell surface.

As shown in Figure 9, inhibition of myosin light chain kinase attenuates VCAM expression, although it is ineffective in reducing the surface expression of the other CAMs. Inhibition of Rho kinase does not alter the expression of any of the CAMs. Both myosin light chain kinase [Zhao and Davis, 1999] and Rho kinase [Wojciak-Stothard et al., 1998] are important in actin filament generation and reorganization of the cytoskeleton. The concentrations of ML-7 and Y27632 we used have been shown to completely inhibit myosin light chain phosphorylation and stress fiber formation in other instances [Garcia et al., 1995; Katoh et al., 2001; Petrache et al., 2001; and except for ML-7s lack of effect on myosin light chain phosphorylation, in this study]. However, myosin light chain kinase and Rho kinase appear to work on different actin structures. There are two distinct pools of stress fibers in non-muscle cells. The cortical stress fibers that are at the cell periphery, and the central stress fibers, which run across the cells. The former are regulated by a myosin light chain kinase-dependent mechanism while the latter depends on Rho kinase [Katoh et al., 2001]. Rho kinase appears to regulate both basal and TNF-α-induced myosin light chain phosphorylation and central stress fiber formation in our cells but Y27632 did not affect CAM surface expression, suggesting that

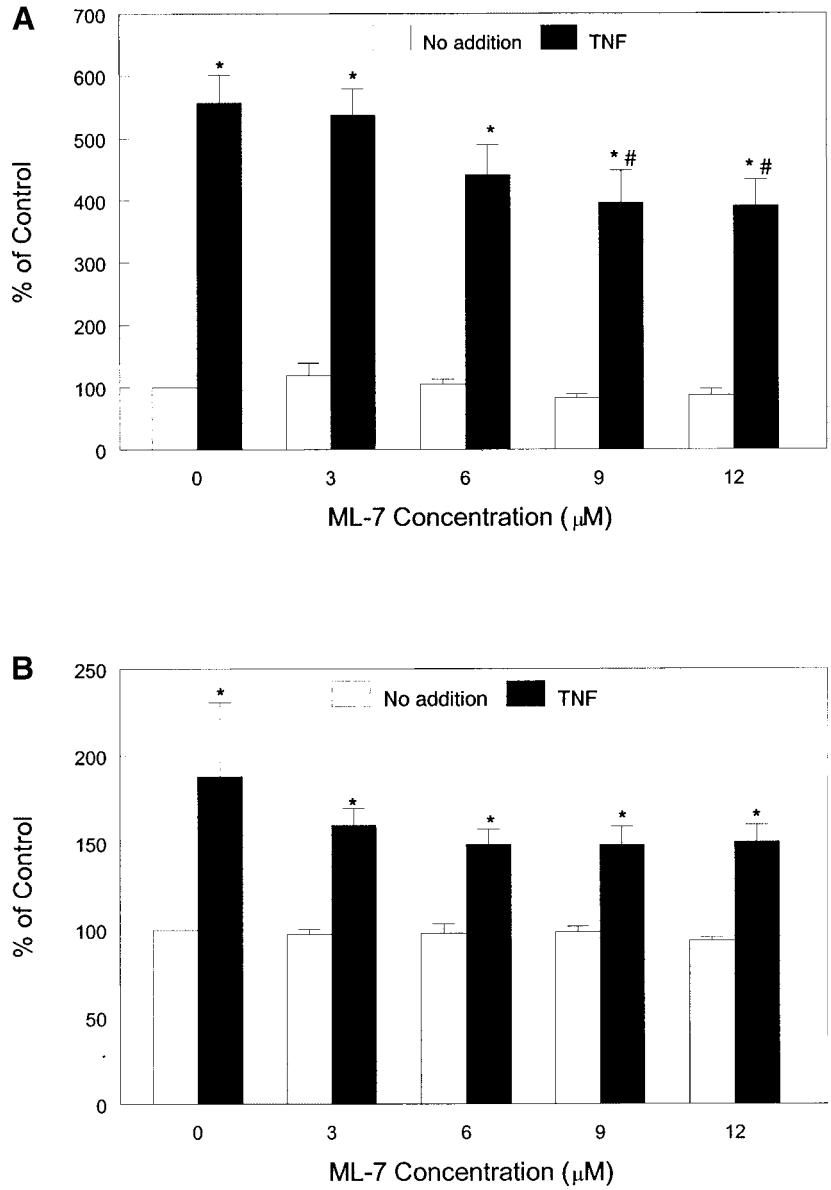

Fig. 9. The effect of ML-7 on cell surface adhesion molecule expression. HAEC were incubated for 30 min with different concentrations of ML-7 then stimulated for 4 h with 20 ng/ml TNF-α. VCAM-1 (**A**), ICAM-1 (**B**), and E-selectin (**C**) were detected as described in Figure 8. Incubation with ML-7, at 9–12 μM, modestly attenuated VCAM-1 expression but had no affect on the expression of ICAM-1 or E-selectin. *, $P < 0.05$, compared to control (no TNF-α) for each inhibitor. #, $P < 0.05$, compared to TNF-α treated, no inhibitor.

the central stress fibers were not necessary for CAM translocation to the membrane. On the other hand, ML-7 modestly inhibited VCAM surface expression and reduced the peripheral cortical actin. However, ML-7 had no affect on myosin light chain phosphorylation. This is puzzling since ML-7 appears to be highly selective for myosin light chain kinase [Bain et al., 2003], although it does suggest that cortical actin stress fibers play an insignificant role in CAM translocation to the cell membrane.

The transcription of the adhesion molecules studied herein is regulated through the NF-κB pathway [Collins et al., 1995; Kim et al., 2001]. Increased transcription may make more protein available for translocation to the cell membrane, however, a functioning actin cytoskeleton appears to play an important role in

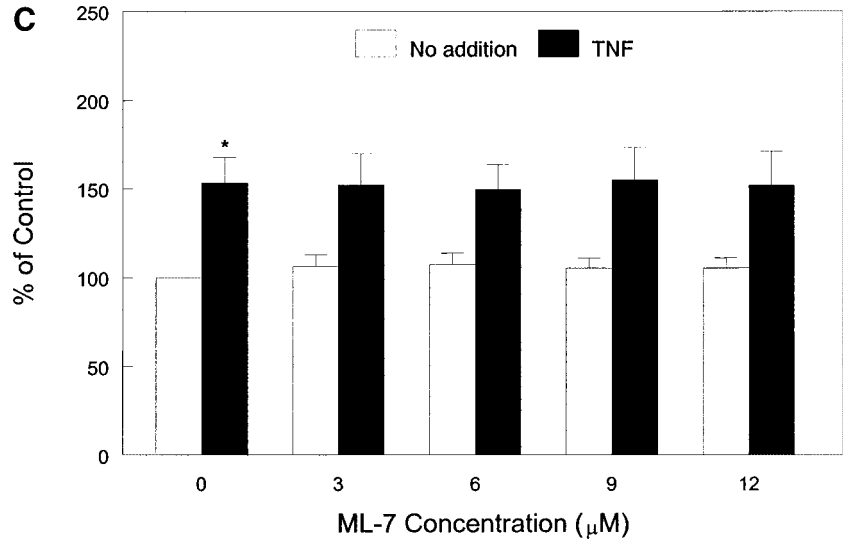

Fig. 9. (*Continued*)

delivering VCAM and ICAM-1 to the cell surface. Nevertheless, there seems to be no selectivity between the specific pools of stress fibers (cortical vs. central) for this translocation. Subtle differences in the cytoskeletal requirements for the translocation of these adhesion molecules are apparent with E-selectin much less dependent on the actin cytoskeleton.

ACKNOWLEDGMENTS

We thank Laura James, MS for help with the statistical analyses and Christina Stamper and Nancy Kleene for technical assistance with the confocal microscope.

REFERENCES

Adelstein RS, Pato MD, Conti MA. 1981. The role of phosphorylation in regulating contractile proteins. Adv Cyclic Nucleotide Res 14:361–373.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271:20246–20249.

Bain J, McLauchlan H, Elliott M, Cohen P. 2003. The specificities of protein kinase inhibitors: An update. Biochem J 371:199–204.

Balaram SK, Agrawal DK, Allen T, Kuszynski CA, Edwards JD. 1997. Cell adhesion molecules and insulin-like growth factor-1 in vascular disease. J Vasc Surg 25:866–876.

Blagoveshchenskaya AD, Hannah MJ, Allen S, Cutler DF. 2002. Selective and signal-dependent recruitment of membrane proteins to secretory granules formed by heterologously expressed von Willebrand factor. Mol Biol Cell 13:1582–1593.

Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. FASEB J 9:899–909.

Deisher TA, Haddix TL, Montogomery KF, Pohlman TH, Kaushansky K, Harlan JM. 1993. The role of protein kinase C in the induction of VCAM-1 expression on human umbilical vein endothelial cells. FEBS Lett 331:285–290.

Deli MA, Descamps L, Dehouch MP, Cecchelli R, Joo F, Abraham CS, Torpier G. 1995. Exposure of tumor necrosis factor-α to luminal membrane of bovine brain capillary endothelial cells cocultured with astrocytes induces a delayed increase of permeability and cytoplasmic stress fiber formation of actin. J Neurosci Res 41: 717–726.

Dudek SM, Garcia JGN. 2001. Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol 91: 1487–1500.

Gao B, Saba TM, Tsan M-F. 2002. Role of $\alpha_v\beta_3$-integrin in TNF-α-induced endothelial cell migration. Am J Physiol 283:C1196–C1205.

Garcia JGN, Davis HW, Patterson CE. 1995. Regulation of endothelial cell gap formation and barrier dysfunction: Role of myosin light chain phosphorylation. J Cell Physiol 163:510–522.

Garcia JGN, Verin AD, Herenyiova M, English D. 1998. Adherent neutrophils activate endothelial myosin light chain kinase: Role in transendothelial migration. J Appl Physiol 84:1817–1821.

Goeckeler ZM, Wysolmerski RB. 1995. Myosin light chain kinase-regulated endothelial cell contraction: The relationship between isometric tension, actin polymerization and myosin phosphorylation. J Cell Biol 130:613–627.

Gotlieb AI, Wong MKK, Boden P, Fone AC. 1987. The role of the cytoskeleton in endothelial repair. Scan Microsc 1:1715–1726.

Hahne M, Jager U, Isenmann S, Hallmann R, Vestweber D. 1993. Five tumor necrosis factor-inducible cell adhesion

mechanisms on the surface of mouse endothelioma cells mediate the binding of leukocytes. J Cell Biol 121: 655–664.

Katoh K, Kano Y, Amano M, Kaibuchi K, Fujiwara K. 2001. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. Am J Physiol 280: C1669–C1679.

Kim I, Moon S-O, Kim SH, Kim HJ, Koh YS, Koh GY. 2001. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1, vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-κB activation in endothelial cells. J Biol Chem 276:7614–7620.

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273:245–248.

Krunkosky TM, Fischer BM, Akley NJ, Adler KB. 1996. Tumor necrosis factor alpha (TNF alpha)-induced ICAM-1 surface expression in airway epithelial cells in vitro: Possible signal transduction mechanisms. Ann NY Acad Sci 796:30–37.

Lampugnani MG, Giorgi M, Gaboli M, Dejana E, Marchisio PC. 1990. Endothelial cell motility, integrin receptor clustering, and microfilament organization are inhibited by agents that increase intracellular cAMP. Lab Invest 63:521–531.

Lane TA, Lamkin GE, Wancewicz EV. 1990. Protein kinase C inhibitors block the enhanced expression of intercellular adhesion molecule-1 on endothelial cells activated by interleukin-1, lipopolysaccharide, and tumor necrosis factor. Biochem Biophys Res Commun 172:1273–1281.

Libby P. 2002. Inflammation in atherosclerosis. Nature 420:868–874.

Madge LA, Pober JS. 2001. TNF signaling in vascular endothelial cells. Exp Mol Pathol 70:317–325.

Matheny HE, Deem TL, Cook-Mills JM. 2000. Lymphocyte migration through monolayers of endothelial cell lines involves VCAM-1 signaling via endothelial cell NADPH oxidase. J Immunol 164:6550–6559.

May MJ, Wheeler-Jones CP, Pearson JD. 1996. Effects of protein tyrosine kinase inhibitors on cytokine-induced adhesion molecule expression by human umbilical vein endothelial cells. Br J Pharmacol 118:1761–1771.

Miranti CK, Brugge JS. 2002. Sensing the environment: A historical perspective on integrin signal transduction. Nat Cell Biol 4:83–90.

Neltner BS, Zhao Y, Sacks DB, Davis HW. 2000. Thrombin-induced phosphorylation of MARCKS does not alter its interactions with calmodulin or actin. Cell Signal 12:71–79.

Petrache I, Verin AD, Crow MT, Birukova A, Liu F, Garcia JGN. 2001. Differential effect of MLC kinase in TNF-α-induced endothelial cell apoptosis and barrier function. Am J Physiol 280:L1168–L1178.

Ross R. 1999. Atherosclerosis: An inflammatory disease. N Engl J Med 340:115–126.

Saito J, Minamiya Y, Kitamura M, Saito S, Enomoto K, Terada K, Ogawa J. 1998. Endothelial myosin light chain kinase regulates neutrophil migration across human umbilical vein endothelial cell monolayer. J Immunol 161:1533–1540.

Saito H, Minamiya Y, Saito S, Ogawa J. 2002. Endothelial Rho and Rho kinase regulate neutrophil migration via endothelial myosin light chain phosphorylation. J Leukoc Biol 72:829–836.

van der Wal AC, Das PK, Tigges AJ, Becker AE. 1992. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. Am J Pathol 141:1427–1433.

Wojciak-Stothard B, Entwistle A, Ridley AJ, Garg R. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell–cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. J Cell Physiol 176:150–165.

Wojciak-Stothard B, Williams L, Ridley AJ. 1999. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. J Cell Biol 145:1293–1307.

Wood KM, Cadogan MD, Ramshaw AL, Parums DV. 1993. The distribution of adhesion molecules in human atherosclerosis. Histopathology 22:437–444.

Zhao Y, Davis HW. 1999. Signaling pathways in thrombin-induced actin reorganization in pulmonary artery endothelial cells. Exp Lung Res 25:23–39.