Activated Neutrophils Induce Hyperpermeability and Phosphorylation of Adherens Junction Proteins in Coronary Venular Endothelial Cells*

(Received for publication, February 19, 1999, and in revised form, May 19, 1999)

John H. Tinsley, Mack H. Wu, Weiya Ma, Amy C. Taulman, and Sarah Y. Yuan‡

From the Departments of Surgery and Medical Physiology, Texas A&M University System Health Science Center, Temple, Texas 76504

The endothelial adherens junction is formed by complexes of transmembrane adhesive proteins, of which β-catenin is known to connect the junctional protein vascular endothelial (VE)-cadherin to the cytoskeleton and to play a signaling role in the regulation of junction-cytoskeleton interaction. In this study, we investigated the effect of neutrophil activation on endothelial monolayer integrity and on β-catenin and VE-cadherin modification. Treatment of cultured bovine coronary endothelial monolayers with C5a-activated neutrophils resulted in an increase in permeability as measured by albumin clearance across the monolayer. Furthermore, large scale intercellular gap formation was observed in coincidence with the hyperpermeability response. Immunofluorescence analysis showed that β-catenin and VE-cadherin staining changed from a uniform distribution in treated cells. Finally, β-catenin and VE-cadherin from neutrophil-treated endothelial cells showed a significant increase in tyrosine phosphorylation. Our results are the first to link neutrophil-mediated changes in adherens junctions with intercellular gap formation and hyperpermeability in microvascular endothelial cells. These data suggest that neutrophils may regulate endothelial barrier function through a process conferring conformational changes to β-catenin and VE-cadherin.

The wall of exchange vessels consists of a layer of endothelial cells that connect to each other with closely opposed intercellular junctions. A major function of the junctional connection is to maintain the semi-permeable property of the endothelial barrier and to control the transvascular passage of solutes, fluid, and blood cells. Four types of junctions associated with endothelial cells have been identified: adherens junctions (AJ), tight junctions, gap junctions, and complexus adherentes (1, 2). AJ, formed by transmembrane adhesive proteins called cadherins, appear to be the main complex regulating macromolecular permeability in microvascular endothelium. Cadherins, specifically vascular endothelial (VE)-cadherin, are associated with the actin cytoskeleton through a family of proteins called catenins, including α-catenin, β-catenin, and plakoglobin (3, 4).

The endothelial permeability is affected by many agonists including α-thrombin, histamine, and phorbol esters (5–9) as well as by a group of inflammatory cells, namely polymorphonuclear leukocytes (PMNs) (10–14). At the site of injury or inflammation, circulating PMNs often adhere to and subsequently migrate through the endothelium and enter surrounding tissues (15). It has long been documented that the process of PMN adherence and migration is associated with an increase in endothelial permeability (10, 11). Although much work has been dedicated to identify PMN-derived hyperpermeability factors (11–14), little is known about the molecular targets of activated PMNs and the derived factors. Within this context, whether PMNs affect microvascular barrier function by altering the structural and functional integrity of the endothelium remains elusive.

Recent evidence suggests that the AJ is involved in PMN-promoted endothelial barrier dysfunction. Upon PMN adhesion, VE-cadherin proteolysis is accompanied by the disappearance of both VE-cadherin and catenins from AJ (14, 16, 17). Another hyperpermeability agonist, vascular endothelial growth factor, has been shown to increase the transendothelial flux of albumin concomitant with a loss of VE-cadherin (18). These studies have provided a possible linkage between PMN activation and VE-cadherin disorganization in the regulation of endothelial barrier function. Furthermore, evidence is accumulating that the VE-cadherin-mediated cell-cell adhesion is controlled by a dynamic balance between phosphorylation and dephosphorylation of the junctional proteins including cadherins and catenins. Increased tyrosine phosphorylation of β-catenin resulted in a dissociation of the catenin from cadherin and from the cytoskeleton, leading to a weak AJ (19, 20). Similarly, tyrosine phosphorylation of VE-cadherin and β-catenin occurred in loose AJ and was notably reduced in tightly confluent monolayers (21).

This study focuses on the effect of activated PMNs on both endothelial permeability and phosphorylation of AJ proteins. The results showed that tyrosine phosphorylation of VE-cadherin and β-catenin was increased when cultured coronary venular endothelial cells (CVECs) were exposed to activated PMNs. β-Catenin and VE-cadherin staining revealed a marked decrease in the amount of these proteins at the cell periphery upon stimulation by PMNs. Correspondingly, PMN-treated monolayers showed a significant increase in permeability as measured by albumin clearance. This work is the first to correlate hyperpermeability and tyrosine phosphorylation of junc-
PMN-induced Hyperpermeability and Phosphorylation

37°C for 45 min. The supernatant was collected and centrifuged at 400
hypotonic hemolysis. PMNs were harvested and washed twice by saline, and lysed on ice for 30 min with 300
Phorbol 12-myristate 13-acetate (PMA) (10⁻⁵ M), or calyculin A (10⁻⁸ M) for 30 min. Fluorescein isothiocyanate-albumin was then added to the luminal chamber, and permeability (Pₜ) was calculated after 45 min. (Pₜ) values were expressed as percentage of control cells. *, p < 0.05.

Cell Culture and Treatment—Bovine CVECs were isolated from post-capillary venules (about 15 μm in diameter) as described previously (22). CVECs were routinely maintained on gelatin-coated dishes containing 10% fetal bovine serum in complete Dulbecco’s modified Eagle’s medium (with 1 mM sodium pyruvate, 2 mM L-glutamine, 15 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 25 units/ml heparin). The cells exhibited properties characteristic of the endothelial cell, such as typical cobblestone morphology, positive immunofluorescent staining for factor VIII antigen, uptake of diacyctylated low density lipoprotein, and the ability to form tubes (22). Cells were used at passages 6–14 and grown to confluence before treatment with drugs: complement 5a (C5a) 10⁻⁷ M and phenylarsine oxide (PAO) 10⁻⁸ M, or hydrocortisone (HCS), 10⁻⁵ M dammaancanthal (DAM), 10⁻⁵ M bisindolylmaleimide I (BIM), 10⁻⁷ M phorbol 12-myristate 13-acetate, 10⁻⁷ M genistein, 10⁻⁷ M Go6976, and 10⁻⁸ M calyculin A (Calbiochem). PMNs were isolated from porcine blood as described below, activated with C5a for 15 min, then added to the CVECs at a concentration of 10⁶/ml.

Isolation of Porcine PMNs—Platelet-poor plasma was obtained by centrifuging 40 ml of plasma at 2500 × g for 10 min. The plasma was added to Hanks’ buffer solution to a concentration of 10% and used as diluent or washing solution for the following procedures. To isolate PMNs, 20 ml of whole blood were diluted with 40 ml of Hanks’ buffer solution, carefully layered on top of a 59% isotonic Percoll column, and centrifuged at 400 × g for 20 min. The top band on the centrifuged Percoll column (containing lymphocytes) was discarded. The pellet containing red blood cells and PMNs was collected and mixed two parts (v/v) of 2.5% gelatin in Hanks’ buffer solution for incubation at 37°C for 45 min. The supernatant was collected and centrifuged at 300 × g for 10 min. The remaining red cell pellet was then removed by hypotonic hemolysis. PMNs were harvested and washed twice by centrifuging at 400 × g for 5 min. From this technique, 20 ml of blood yielded 10⁹-10⁹ cells, of which 90–95% were PMNs. In vitro analyses demonstrated that the isolated PMNs were viable and displayed normal chemotaxis function and metabolic oxygenation activity (10).

Immunoprecipitation and Western Analysis—CVECs were treated as described above for 30 min at 37°C, washed with phosphate-buffered saline, and lysed on ice for 30 min with 300 μl of radioimmune precipitation buffer (1× phosphate-buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mg/ml), aprotonin (1 μg/ml), and sodium orthovanadate (1 mM) (Sigma). After centrifugation for 3 min at 14,000 rpm, cell lysates were removed, and protein concentrations were determined using the Bradford assay. For immunoprecipitation (IP), 100 μg of cell lysate was used in 1 ml of radioimmunoprecipitation buffer. Five μg of antibody against phosphotyrosine PY20 (Santa Cruz Biotechnology, Inc., Santa Cruz, California) was added, and the sam-

![Image](68x578 to 278x729)

**FIG. 1.** Albumin permeability of CVEC monolayers in response to PAO, phorbol 12-myristate 13-acetate, and calyculin A. CVECs were grown to confluence on Transwell membranes and treated with PAO (10⁻⁸ M), phorbol 12-myristate 13-acetate (PMA) (10⁻⁵ M), or calyculin A (10⁻⁸ M) for 30 min. Fluorescein isothiocyanate-albumin was then added to the luminal chamber, and permeability (Pₜ) was calculated after 45 min. (Pₜ) values were expressed as percentage of control cells. *, p < 0.05.

**FIG. 2.** PMNs induced hyperpermeability in CVECs. CVECs were grown to confluence on Transwell membranes and treated for 30 min with control (a), C5a (b), PMNs (c), PMNs/C5a (d), DAM (e), PMNs/C5a/DAM (f), BIM (g), PMNs/C5a/BIM (h), genistein (i), PMNs/C5a/Genistein (j), Go6976 (k), and PMNs/C5a/Go6976 (l). Albumin clearance across the membrane was measured following calculation of the permeability coefficient. Data are shown as the percentage of control. *, p < 0.05.
PMN-induced Hyperpermeability and Phosphorylation

Fig. 3. PMN-induced tyrosine phosphorylation of β-catenin. CVECs were treated with PMNs and drugs for 30 min. In A, protein levels reflect total cell lysate, and phosphorylation levels were obtained after IP with a phosphotyrosine antibody. Both Westerns were probed with a β-catenin antibody. In B, the phosphorylation levels were quantitated by scanning densitometry and comparing phosphorylation levels to total β-catenin levels. For both A and B, results are shown with control (a), C5a (b), PMNs (c), PMNs/C5a (d), DAM (e), PMNs/C5a/DAM (f), BIM (g), PMNs/C5a/BIM (h), and PAO (i). *, p < 0.05. O.D., optical density.

Respect to microvascular permeability, it was necessary to adapt a model previously used with large vascular endothelial cells for use with our venular endothelial cells. Our preliminary studies revealed that coating the polycarbonate membranes with gelatin was superior to fibronectin, and the gelatin imposed no major restriction to albumin clearance. Membranes with a gelatin coating and no cells had a 14-fold higher 

PMN-induced Hyperpermeability and Phosphorylation

Fig. 4. PMN-induced tyrosine phosphorylation of VE-cadherin. CVECs were treated with PMNs and drugs for 30 min. In A, protein levels reflect total cell lysate, and phosphorylation levels were obtained after IP with a phosphotyrosine antibody. Both Westerns were probed with a VE-cadherin antibody. In B, the phosphorylation levels were quantitated by scanning densitometry and comparing phosphorylation levels to total VE-cadherin levels. For both A and B, results are shown with control (a), C5a (b), PMNs (c), PMNs/C5a (d), DAM (e), PMNs/C5a/DAM (f), BIM (g), PMNs/C5a/BIM (h), and PAO (i). *, p < 0.05. O.D., optical density.

Both DAM and genistein, tyrosine kinase inhibitors, and BIM and Go6976, serine kinase inhibitors. Additionally, BIM seemed to cause a decrease in the basal permeability, but the significance of this effect has not been determined.

Activated PMNs Induce Tyrosine Phosphorylation of Endothelial Junctional Proteins—Knowing that PMNs are involved in hyperpermeability processes involving tyrosine and serine phosphorylation events, we looked for changes in phosphorylation levels of proteins found at intercellular junctions and, therefore, suspected of regulating endothelial integrity. Fig. 3 shows that activated PMNs increase tyrosine phosphorylation of β-catenin ~225% above control levels. As expected, this increase could be attenuated by the addition of a tyrosine kinase inhibitor (DAM); however, we also somewhat unexpectedly observed an attenuation of this increase with the addition of a serine kinase inhibitor (BIM) (Fig. 3). Another AJ protein, VE-cadherin, showed an ~100% increase in tyrosine phosphorylation content after exposing CVECs to activated PMNs (Fig. 4). As with β-catenin, the increase in tyrosine phosphorylation of VE-cadherin brought on by activated PMNs was attenuated with DAM and BIM (Fig. 4). These results suggest an interaction between tyrosine and serine phosphorylation events as they relate to AJ proteins and monolayer permeability. Additionally, C5a-activated PMNs appear to be a more potent stimulator of AJ protein tyrosine phosphorylation than the potent tyrosine phosphatase inhibitor PAO (Figs. 3 and 4).

Distribution of β-Catenin, VE-cadherin, and Actin in Response to Activated PMNs—In view of the result that activated PMNs induced phosphorylation either directly or indirectly of two major AJ proteins, we wanted to determine if changes in
cellular distribution accompanied this hyperphosphorylation. Confluent CVEC monolayers were exposed to activated PMNs and then subjected to immunocytochemistry to detect changes in β-catenin and/or VE-cadherin localization. In addition, because VE-cadherin is associated with the actin cytoskeleton through the catenins, we examined actin distribution in PMN-treated cells. In control cells, β-catenin is uniformly dispersed around the periphery of a tightly confluent monolayer (Fig. 5A). When the cells are treated with activated PMNs, we see widespread gap formation as the cells retract and a loss of β-catenin staining in areas where individual cells have lost contact with neighboring cells (Fig. 5B). Note also that β-catenin assumes a finger-like configuration between these minimal cell contacts (Fig. 5B). VE-cadherin shows a peripheral staining pattern in control CVEC monolayers (Fig. 5C). However, PMN-treated cells show widespread gap formation and a diffuse staining pattern for VE-cadherin, leaving little if any VE-cadherin at the cell periphery (Fig. 5D). We surmised that for cells to retract in response to PMNs, there are probably accompanying changes in the actin cytoskeleton. Actin staining of control cells revealed that most of the F-actin was located at the cell periphery (Fig. 5E). However, in PMN-treated cells, we consistently observed an increase in stress fiber formation (Fig. 5F), which indicates cell contraction and shape change. In all of the PMN-treated endothelial monolayers, adherent PMNs were often observed where intercellular gaps were seen (Fig. 5B, arrow).

DISCUSSION

PMN-induced vascular leakage has long been implicated in the development of coronary microvascular dysfunction during ischemia-reperfusion injury and other types of cardiovascular disorders (12, 13, 24–27). Our study is the first systematic examination of the direct effects of PMN activation on the function and structure of coronary microvascular endothelial cells. We had previously demonstrated the interaction of PMNs with the endothelium using intact, perfused coronary venules and arterioles (10). We modified existing monolayer models (12, 13, 18) to accommodate CVECs to study processes occurring at the molecular level in these cells. Compared with the intact, perfused coronary venules, the CVEC monolayer exhibited a higher basal permeability but a similar responsiveness to the hyperpermeability stimulators.

The complexity of the precise molecular events that lead to

FIG. 5. Immunofluorescent staining of β-catenin, actin, and VE-cadherin in CVECs. Cells were grown to confluence and treated with C5a-activated PMNs for 30 min. Panels A, C, and E represent control staining for β-catenin, VE-cadherin, and actin, respectively. Panels B, D, and F represent β-catenin, VE-cadherin, and actin staining after PMN treatment. In panels B and D, note the gap formation and absence of β-catenin and VE-cadherin in areas where the cells have separated. The arrow in panel B points to a PMN adhered to the monolayer. In panel F, note the increase in stress fiber formation in the cells compared with control actin staining in panel E.
microvascular leakage is becoming evident. To allow large molecules and even blood cells to pass through the endothelial layer, individual endothelial cells must contract to form gaps. In this process, the intercellular junction may undergo conformational changes, facilitating the gap formation and macromolecular transflux. VE-cadherin, a transmembrane protein, and β-catenin, a signaling protein that links cadherins to the actin cytoskeleton, were found to exhibit increases in tyrosine phosphorylation levels concomitant with an increase in permeability. We suggest that this phosphorylation is an important signal responsible for breaking down the AJ and diminishing the ability of neighboring cells to interact. Our results and those of others showed the disappearance of AJ proteins at the cell periphery under conditions of hyperpermeability (14, 16, 17). Additionally, we observed an increase in the formation of actin stress fibers that coincides with the AJ breakdown and permeability increases. In PMN-treated monolayers, although not all cells show stress fiber formation, there is an overall increase in stress fiber formation across the monolayer versus control cell monolayers. Presumably these stress fibers make contact with opposite sides of the cell membrane and “pull” the cell into a spherical shape, breaking the contacts between neighboring cells. The question then becomes, to what protein or protein complex are the actin fibers anchoring during hyperpermeability situations? Our hypothesis is that AJ proteins anchor the peripheral actin filaments under resting conditions and allow for the integrity of the monolayer to remain intact. In response to agonists, actin stress fibers form and interact with structure(s) including the AJ to instigate changes in cellular morphology, leading to a weak or diminished AJ. Phosphorylation of VE-cadherin and β-catenin may be a critical signaling event that elicits such structural changes. In further support of this, our data show that inhibiting phosphorylation of VE-cadherin and β-catenin blocks the hyperpermeability response in the endothelial cells.

The precise mechanism by which PMNs increase microvascular permeability has not been established. A body of evidence supports that the leakage is mainly attributed to PMN-derived cytotoxic mediators including oxygen radicals and various proteases (28–31). At the site of PMN adhesion, large amounts of the cytotoxic factors are released and accumulate, initiating a full cascade of signaling reactions in the endothelium. Ultimately, endothelial cell contraction occurs, and intercellular gaps are formed. Based on our previous and present studies, it appears that protein phosphorylation is one of the signaling events that is activated in response to PMN adhesion, and these kinase cascades result in the hyperpermeability response. In support of this, we found that activated PMNs induce increases in tyrosine phosphorylation of AJ proteins accompanied by an increase in permeability. Interestingly, we also discovered that serine kinase inhibitors could attenuate the permeability increase provoked by PMNs, and a serine phosphatase inhibitor increased basal permeability. However, our attempts to detect serine phosphorylation of β-catenin and VE-cadherin were unsuccessful. It is likely that activated PMNs initiate events that directly or indirectly up-regulate both the tyrosine and serine phosphorylation pathways, which may interact (cross-talk) with each other. Our opinion is that both types of phosphorylation are necessary to instigate hyperpermeability of CVEC monolayers.

This study is the first to correlate PMN activation with phosphorylation of AJ proteins and hyperpermeability in cultured microvascular endothelial monolayers. We had previously reported that tyrosine phosphorylation of the focal adhesion proteins Paxillin and focal adhesion kinase occurs in CVEC monolayers in conjunction with a breakdown of endothelial barrier function (23). Therefore, we now have evidence that alterations in both the focal adhesion complex and the AJ can result in hyperpermeability of the microvascular endothelium and that activated PMNs can induce at least some of these events. We have established the appropriate model for further studies aimed at methodically dissecting the molecular events associated with changes in CVEC permeability.

REFERENCES

1. Rubin, L. L. (1992) Cell Biol. 4, 830–833
2. Dejana, E., Corada, M., and Lampugnani, M. G. (1995) FASEB J. 9, 910–918
3. Kemper, E. (1995) Trends Genet. 9, 317–321
4. Lampugnani, M. G., Corada, M., Caveda, L., Breviario, F., Ayala, O., Geiger, B., and Dejana, E. (1995) J. Cell Biol. 129, 203–217
5. Ehinger, W. D., Edwards, M. J., and Miller, P. N. (1996) J. Cell. Physiol. 167, 562–569
6. Drake, W. T., Lopes, N. N., Fenton, J. W., II, and Issekutz, A. C. (1992) Lab. Invest. 67, 617–627
7. Schaeffer, R. C., Gong, F., Bitrick, M. S., and Smith, T. L. (1993) Am. J. Physiol. 264, H1798–H1809
8. Yuan, Y., Granger, H. J., Zawieja, D. C., DeFilipps, D. V., and Chilian, W. M. (1993) Am. J. Physiol. 264, H1734–H1739
9. Huang, Q., and Yuan, Y. (1997) Am. J. Physiol. 273, H2442–H2451
10. Yuan, Y., Mier, R. A., Chilian, W. M., Zawieja, D. C., and Granger, H. J. (1995) Am. J. Physiol. 268, H480–H488
11. Beynon, H. L., Davies, K. A., Haskard, D. O., and Walport, M. J. (1994) J. Immunol. 153, 3160–3167
12. Gibbins, L. S., Lai, L., and Malik, A. B. (1990) J. Cell. Physiol. 145, 496–500
13. Rosengren, S., Oldsson, A. M., von Andrian, U. H., Lundgren-Akerlund, E., and Arfors, K. E. (1991) J. Appl. Physiol. 71, 1322–1330
14. Carden, D., Xiao, F., Moak, C., Willis, B. H., Robinson-Jackson, S., and Alexander, S. (1998) Am. J. Physiol. 275, H385–H392
15. Burns, A. R., Walker, D. C., Brown, E. S., Thurmon, L. T., Bowden, R. A., Keese, C. R., Simon, S. I., Entman, M. L., and Smith, C. W. (1997) J. Immunol. 159, 2893–2903
16. Del Maschio, A., Zanetti, A., Corada, M., Rival, Y., Baro, L., Lampugnani, M. G., and Dejana, E. (1996) J. Cell Biol. 135, 497–510
17. Allport, J. R., Ding, H., Collins, T., Gerritsen, M. E., and Luscinskas, F. W. (1997) J. Exp. Med. 186, 1517–1521
18. Keivi, C. G., Payne, D. K., Mires, E., and Alexander, J. S. (1998) J. Biol. Chem. 273, 15099–15103
19. Krypa, R. M., Su, H., and Reichardt, L. F. (1996) J. Cell Biol. 134, 1519–1529
20. Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S., and Takeichi, M. (1992) J. Cell Biol. 118, 703–714
21. Lampugnani, M. G., Corada, M., Andriopoulos, P., Eser, S., Risau, W., and Dejana, E. (1997) J. Cell Biol. 139, 2065–2077
22. Schelling, M. E., Meiringer, C. J., Hawker, J. R., Jr., and Granger, H. J. (1998) Am. J. Physiol. 354, H2121–H2127
23. Yuan, Y., Meng, Y. F., Huang, Q., Hawker, J. W., and Wu, H. M. (1998) Am. J. Physiol. 275, H884–H893
24. Huang, A. J., Manning, J. E., Bandak, T. M., Ratau, M. C., Hansen, K. R., and Silverstein, S. C. (1993) J. Cell Biol. 120, 1371–1380
25. Blessey, J. E., Pearson, J. D., Carlton, J. S., Hutchings, A., and Gordon, J. L. (1978) J. Cell Sci. 33, 85–101
26. Furie, M. B., Nappstek, B. L., and Silverstein, S. C. (1987) J. Cell Sci. 88, 461–475
27. Taylor, R. F., Price, T. H., Schwartz, S. M., and Dale, D. C. (1981) J. Clin. Invest. 67, 584–587
28. Sepper, R., Kontinen, Y. T., Ingman, T., and Sorsa, T. (1995) J. Clin. Immunol. 15, 27–34
29. Smalley, L. A., Tenncese, M. G., Sandhaus, R. A., Haslett, C., Guthrie, L. A., Johnston, R. B., Henson, P. M., and Worthen, G. S. (1986) J. Clin. Invest. 77, 1233–1243
30. Sutter, N., Nolte, A., Wilke, A., and Drenckhahn, D. (1993) Int. J. Microcirc. Clin. Exp. 13, 187–203
31. Weiss, S. J., and Regian, S. (1984) J. Cell. Biol. 73, 1297–1303