Oxidant stress and endothelial cell dysfunction

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Lum, Hazel, and Kenneth A. Roebuck. Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol 280: C719–C741, 2001.—Reactive oxygen species (ROS) are generated at sites of inflammation and injury, and at low levels, ROS can function as signaling molecules participating as signaling intermediates in regulation of fundamental cell activities such as cell growth and cell adaptation responses, whereas at higher concentrations, ROS can cause cellular injury and death. The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissues, is a major target of oxidant stress, playing a critical role in the pathophysiology of several vascular diseases and disorders. Specifically, oxidant stress increases vascular endothelial permeability and promotes leukocyte adhesion, which are coupled with alterations in endothelial signal transduction and redox-regulated transcription factors such as activator protein-1 and nuclear factor-kB. This review discusses recent findings on the cellular and molecular mechanisms by which ROS signal events leading to impairment of endothelial barrier function and promotion of leukocyte adhesion. Particular emphasis is placed on the regulation of cell-cell and cell-surface adhesion molecules, the actin cytoskeleton, key protein kinases, and signal transduction events.

endothelial permeability; leukocyte transmigration; actin filament; cadherin; occludin; intercellular adhesion molecule-1; selectins; redox-sensitive signal transduction; nuclear factor-kB

ACUTE AND CHRONIC OXIDANT STRESS to the vascular endothelium is a serious causative factor of vascular endothelial dysfunction and plays an important role in the pathophysiology of several vascular diseases, including atherosclerosis, diabetes, neuronal disorders, and ischemia-reperfusion injury. Two aspects of endothelial dysfunction may be particularly important in determining the severity of a vascular disorder: 1) increased endothelial permeability and 2) increased endothelial adhesion for leukocytes, both critical factors governing tissue edema formation and leukocyte extravasation. Reactive oxygen species (ROS) are well documented to function as signaling molecules, stimulating cellular activities ranging from cytokine secretion to cell proliferation, and at higher concentrations, they can induce cell injury and death by oxidant modification of proteins and carbohydrates, lipid peroxidation, and DNA strand nicks. Such diverse responses are related to multiple factors, such as the ROS prevailing at the inflammatory locus, concentration and turnover of the oxidants, and the antioxidant capacity of the local environment as well as target cells. There are several excellent reviews regarding the signaling role of ROS and possible mechanisms of endothelial injury (132, 164). Therefore, this review is specifically focused on recent findings regarding cellular and molecular activities by which ROS signal the impairment of endothelial barrier function and promotion of leukocyte adhesion and extravasation.

EFFECTS OF OXIDANT STRESS ON VASCULAR ENDOTHELIUM

At sites of inflammation and infection, the local cellular environment is enriched with cytokines, chemokines, and ROS. During this period, the endothelium can be exposed to high levels of multiple species of ROS for a prolonged period of time. In the in vivo setting,
subpopulations of polymorphonuclear neutrophils (PMN) are found adherent to the endothelium or migrating through the extravascular tissue matrix. A major source of ROS is blood leukocytes that become activated and adherent to the endothelial cell surface. In vitro studies demonstrate that suspensions of PMN (10^6), when activated with phorbol esters, release ~35–360 nmol O_2^- /h (146, 226). Evidence indicates that adherence to matrix proteins (i.e., laminin, fibronectin, vitronectin) or endothelium primes the PMN for a massive respiratory burst lasting 1–3 h in response to tumor necrosis factor-α (TNF-α) and the chemoattractant formylmethionyl leucylphenylalanine (fMLP) (166). Thus cytokines at the inflammatory site may contribute significantly to the generation of ROS, particularly by PMN.

The activation of endothelial cells also generates ROS and therefore may be a significant contributor in maintaining the oxidant-rich environment at the inflammatory locus. The treatment of human umbilical vein endothelial cells with the cytokines interleukin-1 (IL-1) and interferon-γ (IFN-γ) results in dose- and time-dependent increases in O_2^- (153). Also, vasoactive peptides such as bradykinin can induce the production of O_2^- (40 nmol/2 × 10^6 cells) within 5 min of ligand binding to its receptor on endothelial cells (96). Exposure of endothelial cells to hypoxia followed by reoxygenation induces the production of 10–50 nmol O_2^- /h (144, 257), suggesting that endothelium-derived ROS contribute to ischemia-reperfusion injury.

With the generation of O_2^-, other more reactive intermediates are also produced by the spontaneous or enzymatically catalyzed dismutation of O_2^-:

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]  

(1)

In the presence of transition metals (e.g., Fe^{3+}), O_2^- reduces Fe^{3+} to Fe^{2+} by the iron-catalyzed Haber-Weiss reaction (Eq. 2). The reduced ferrous iron reacts with H_2O_2 via the Fenton reaction (Eq. 3) to generate the highly reactive hydroxyl radical -OH:

\[ O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \]  

(2)

\[ H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^- \]  

(3)

Furthermore, O_2^- may also react with nitric oxide (NO^-), a reactive nitrogen species produced by endothelial cells (123), to form peroxynitrite, which decomposes to form the potent ROS -OH and NO_3^- (21).

Several experimental models of oxidant stress have demonstrated increased leukocyte extravasation. The perfusion of the rat pancreatic vasculature with xanthine/xanthine oxidase resulted in increased leukocyte recruitment as measured by myeloperoxidase assay (100). Similarly, superfusion of the rat mesentery microvasculature with H_2O_2 caused increased transendothelial migration of leucocytes as detected by intravital microscopy (200). In an in vitro model, treatment of human umbilical vein endothelial cells with tert-butylhydroperoxide resulted in a twofold increase in transendothelial migration of monocyte-like HL-60 cells (190).

There is substantive evidence that oxidant stress increases vascular endothelial permeability. The perfusion of ROS (e.g., H_2O_2) or xanthine/xanthine oxidase (which generates O_2^-) into isolated lungs of guinea pigs (110) and rabbits (18, 205) results in increased capillary filtration coefficient within 30 min, leading to pulmonary edema. Similarly, superfusion of ROS onto the surface of brain pial venules results in dose-dependent decreases in transendothelial electrical resistance, indicating barrier impairment (175). In some studies, the increased permeability is accompanied by increased capillary pressure (17, 110), suggesting that both factors may be important in promoting tissue edema formation. The oxidant-mediated vasopressor effect appears to be associated with activation of tyrosine kinases (108), although the definitive signaling mechanisms responsible are not clear. However, other studies have indicated that edema formation in response to oxidant stress can occur in the absence of increased capillary pressure (17, 18, 205). These observations indicate that oxidant-mediated tissue edema may likely be attributed predominantly to increased vascular endothelial permeability and that an accompanying capillary pressure rise may function to accelerate the transvascular fluid flux.

This notion is further supported by in vitro studies in which direct treatment of cultured endothelial cell monolayers with ROS increased transendothelial permeability. The incubation of endothelial monolayers with xanthine/xanthine oxidase (208) or glucose/glucose oxidase (which generates H_2O_2) (97) increased the transendothelial flux of tracer albumin; the duration and severity of the increase corresponded positively with increased ROS generation. Furthermore, direct addition of H_2O_2 increased albumin flux and decreased transendothelial electrical resistance (172, 214). Interestingly, comparison of the effects of H_2O_2 with those of hypochlorous acid (HOCl), a more reactive oxidant than H_2O_2, showed that HOCl causes a faster and greater increase in albumin permeability (172).

The vascular endothelial barrier dysfunction is well documented in ischemia-reperfusion injury, which is associated with toxic amounts of ROS generated primarily by activated leukocytes and endothelial cells. In experimental models of brain ischemia-reperfusion injury, the treatment with a permeant H_2O_2 scavenger and sodium tungstate (which inactivates xanthine oxidase) (177) reduced brain edema, and treatment with polyethylene glycol superoxide dismutase or polyethylene glycol catalase attenuated the increased blood-brain transfer of [14C]urea (11). Similarly, perfusion of superoxide dismutase or a xanthine oxidase inhibitor (allopurinol) protected against increases in vascular permeability in ischemia-reperfusion models of the rabbit lung (99, 116) and hamster cheek pouch (51).
The cadherin cytoplasmic domain, containing the binding site for β-catenin, which links to the actin cytoskeleton (111), may also be a critical region in conferring a restrictive barrier for high-molecular-weight molecules. Using Chinese hamster cells (which lack cadherin and have high monolayer permeability), Navarro and coworkers (167) made stable transfectants expressing either wild-type or mutant VE-cadherin with truncation of the cytoplasmic domain. The wild-type transfectants had lower permeability that was comparable to that of endothelial cells, whereas mutant transfectants had high permeability. However, cell-cell aggregation was not impaired in either transfectant (167). Interestingly, transfection of a carboxy terminus-truncated N-cadherin also did not impair cell-cell adhesion or aggregation (59). These findings suggest that the cadherin amino terminus is required for cell-cell adhesion, whereas the carboxy terminus may function to promote a tight restrictive barrier by anchorage to the actin cytoskeletal network.

Relation of Impaired Cell Junctions to Barrier Dysfunction

At present, there is limited information regarding the effects of oxidant stress on the function and organization of adherens and tight junctional proteins. In one study, in which rat lungs were perfused with H$_2$O$_2$, the increased extravasated FITC-albumin was not associated with altered distribution of ZO-1 as detected by immunogold localization (182). However, in an in vitro study, H$_2$O$_2$ treatment of human umbilical vein endothelial cells resulted in the redistribution of occludin and dissociation from ZO-1, which was associated with phosphorylation on serine residues of occludin (119). Interestingly, Kevil et al. (117) reported that H$_2$O$_2$ treatment of endothelial cells promotes cadherin internalization as a possible mechanism for reducing cadherin expression. Thus disruption of endothelial junctions occurs during oxidant stress and likely provides the basis for increased endothelial permeability (Fig. 1B).

The impairment in expression and organization of the adherens and tight junctional proteins in response to ROS shows features similar to those caused by other inflammatory mediators. For example, histamine (69) or TNF-α (23) can cause reduction or fragmentation of ZO-1 distribution on the cell periphery of microvascular endothelial cells. With adherens junctions, perfusion of rat mesentery venules with TNF-α combined with IFN-γ results in increased albumin permeability and disorganization of VE-cadherin at the junctional site (247). The functional significance of cadherin in barrier function was further investigated with the use of ECV304 cells, which are bladder carcinoma cells lacking cadherin. In this study, ECV304 were transfected with cDNA encoding cadherin-5 or E-cadherin, and the transendothelial resistance in response to histamine was determined. The results indicated that the histamine-mediated decreased resistance occurs pre-
dominantly at the cell-cell junction and not the cell-matrix junction (Fig. 2) (242). These are the first reports establishing a direct functional relationship between cadherin and mediator-induced endothelial permeability changes.

**Actin Filaments in Barrier Function Regulation**

The actin filaments of vascular endothelium are organized into a diffuse network of short microfilaments localized in the cell cortex and into several types of prominent microfilament bundles. These bundles of microfilaments in in vitro cell cultures and in vivo vessels undergo dynamic changes in response to physiological and pathological stresses, including shear stress, vascular pressure, and wound healing processes (62, 241, 244, 246). Increases in endothelial permeability in response to edemagenic mediators are accompanied by intercellular gap formation, cell shape change, and reorganization of actin microfilament bundles. The typical morphological pattern of actin reorganization associated with endothelial barrier dysfunction is increased stress fiber density and reduction or loss of the cortical actin band (also designated as the dense peripheral band) (25, 50, 110, 143). Furthermore, in studies in which endothelial cells were treated with cytochalasins, which depolymerize actin filaments, endothelial permeability was increased (120, 209), whereas in cells treated with phallacidin compounds, which stabilize actin filaments, mediator-induced increases in endothelial permeability were diminished (5, 181). These studies clearly suggest that regulation of endothelial barrier function is dependent on the...
highly dynamic network of actin filaments. Although it is not known precisely how actin may modulate endothelial barrier function, some possible cellular sites of regulation include actin-myosin contraction, intercellular junction assembly/disassembly, cell-matrix adhesion, and transmembrane signaling.

Role of stress fibers and cortical filaments. The actin filaments of both the cortical actin band and stress fibers are associated with α-actinin, myosin, and troponyosin (76); thus the overall cell contraction may engage both filament networks. There is evidence that stress fiber formation is dependent on myosin light chain (MLC) phosphorylation, which activates myosin ATPase, leading to conformational changes that promote myosin filament assembly. These events result in cross-linking of filamentous actin, increased contractile force generation, which drives the formation of stress fibers, and focal adhesions (27). Although there is evidence supporting a role of endothelial contraction in regulation of endothelial barrier function (66, 73, 163), it is not yet clear how this contraction-driven stress fiber formation is utilized by the endothelium to alter permeability. The finding that actin microfilaments of the cortical actin band are linked directly to junctional sites suggests that contractile forces generated from these filaments may somehow affect junctional organization and function. However, this hypothesis remains to be tested.

Table 1. Oxidant stress remodels endothelial actin filament network

<table>
<thead>
<tr>
<th>Endothelial Cell Type</th>
<th>Oxidant Stress</th>
<th>Actin Filament Network</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAEC</td>
<td>Xanthine/xanthine oxidase</td>
<td>Disruption of actin filaments</td>
<td>208</td>
</tr>
<tr>
<td>CPAEC</td>
<td>0–20 mM H₂O₂</td>
<td>Intercellular gap formation</td>
<td>92</td>
</tr>
<tr>
<td>BAEC</td>
<td>0–10 mM H₂O₂</td>
<td>Increased stress fibers</td>
<td>110</td>
</tr>
<tr>
<td>BPMEC</td>
<td>95% O₂</td>
<td>Decreased cortical actin band</td>
<td>180</td>
</tr>
<tr>
<td>HAEC</td>
<td>Reoxygenation</td>
<td>Decreased cortical actin band</td>
<td>172</td>
</tr>
<tr>
<td>BAEC</td>
<td>10 and 100 µM H₂O₂</td>
<td>Increased total F-actin</td>
<td>42</td>
</tr>
<tr>
<td>BPMEC</td>
<td>Reoxygenation</td>
<td>Increased actin to cell periphery</td>
<td>140</td>
</tr>
<tr>
<td>CPAEC</td>
<td>0–0.5 mM H₂O₂</td>
<td>Increased stress fibers</td>
<td>144</td>
</tr>
<tr>
<td>CPAEC</td>
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<tr>
<td>BPMEC</td>
<td>Reoxygenation</td>
<td>Increased actin to cell periphery</td>
<td>213</td>
</tr>
</tbody>
</table>

PPAEC, porcine pulmonary artery endothelial cell; CPAEC, bovine pulmonary artery endothelial cell; HAEC, human aorta endothelial cell; BAEC, bovine aorta endothelial cell; BPMEC, bovine microvascular artery endothelial cell.
Increasing evidence supports a relationship between the endothelial barrier dysfunction and the loss or reduction of the cortical actin band. One important function of the cortical actin band is the assembly and localization of adherens junctions to the lateral cell border. In epithelial cells, treatment with cytochalasins or expression of V12rac (constitutively activated rac) has resulted in disruption of the cortical actin band, accompanied by redistribution of cadherin-catenin protein localization at the cell border to perinuclear Golgi vesicles (184). The reformation of cortical actin band resulted in the return of the cadherin-catenin proteins to the cell border (184). In endothelial cells, sphingosine-1-phosphate, a specific phospholipid ligand of the endothelial differentiation gene receptor proteins, causes dramatic increases in actin stress fibers and cortical actin band, which are associated with localization of VE-cadherin and α-, β-, and γ-catenins to cellular junctions (135). Furthermore, with the expression of mutant forms of RhoA and Rac, the sphingosine-1-phosphate-mediated promotion of actin filaments and junctions was inhibited (135). These observations suggest that the localization of adherens junctions to the cell border is highly dependent on functional actin filament bundles, particularly the cortical actin band. Thus a conceivable mechanism by which edemagenic mediators increase paracellular permeability is via disruption of the cortical actin band, leading to disassembly and loss of the junctions from the cell border. This hypothesis is consistent with reports of loss or disruption of the adherens and tight junctional proteins after exposure to a variety of edemagenic mediators, including histamine, TNF-α, vascular endothelial growth factor (VEGF), and thrombin.

**Actin-binding proteins.** The dynamic assembly and organization of actin filaments into three-dimensional structures that are fundamental for cell activities are controlled by actin-binding proteins (222). Actin-binding proteins are regulated by a multitude of signaling molecules, including phosphoinositides (60, 158), cAMP-dependent protein kinase (87, 107), protein kinase C (PKC) (179, 220), and Ras-related small GTPases (173). Several other actin-binding proteins such as profilin (217), α-actinin (60), and vinculin (71) also bind to and are regulated by phosphatidylinositol 4,5-bisphosphate (PIP2). Furthermore, the Rho GTPases appear to be the underlying foundation for the remod-
eling of the actin filament network. Several actin-binding proteins and enzymes that regulate actin-binding proteins have been identified to be targets of the Rho GTPases (14). For example, LIM kinase catalyzes the phosphorylation and thereby inactivates cofilin, an actin-depolymerizing protein, in a rac-dependent manner, resulting in accumulation of filamentous actin at the cell periphery (252). A direct target of RhoA is Rho-kinase, which phosphorylates Thr-564 of the ezrin/radixin/moesin proteins, interfering with their actin filament/plasma membrane cross-linking function (154). In addition, one of the isoforms (PIP5K) responsible for formation of PIP2 is regulated by RhoA (13). It will be important to fully understand the mechanisms by which the Rho GTPases are regulated and, in turn, how they regulate actin dynamics in the endothelial cell.

Tensegrity model. The coupling of the actin cytoskeleton network to cell shape changes has been explained on the basis of the “cellular tensegrity” concept (106), and therefore this conceptual model may provide some understanding of the mechanical forces driving the cell shape changes associated with increases in endothelial permeability. The model is based on the premise that the dynamic cytoskeleton is an interconnected continuous network that can transmit tension. Cell-generated tensile forces, such as those generated by actin-myosin, are transmitted as tension (centripetally directed) to discrete resistive elements (e.g., cellular junctions, focal adhesions) that oppose this tension. Thus the balance between tensile and resistive forces determines the overall cell shape. However, a change in the tensile or resistive tension shifts this cellular balance of forces, resulting in integrated changes in cell shape, that is, the cell becomes more flattened or rounded. For example, a decrease in the resistive force (e.g., through loss of cell-cell adhesion) would shift the balance of cellular forces to favor tensile forces, resulting in more rounding of the cell. In endothelial cells, there is no clear relationship between cell shape change per se and barrier function; it is known only that shape changes do occur in association with altered permeability. However, the tensegrity model points to the importance of the actin cytoskeleton as a key integrator of global cellular forces, which must somehow impact on all phases of the permeability response.

Oxidants Remodel Actin Filament Network

Endothelial cells respond to oxidant stress with striking changes in their cell shape and remodeling of the actin filament network (Table 1 and Fig. 1). Direct treatment of cultured endothelial cells with H2O2 or xanthine/xanthine oxidase consistently results in cell rounding and formation of intercellular gaps (92, 172, 208, 213). Evidence to date indicates that the oxidant-mediated endothelial cell shape change occurs in association with increased density of actin stress fibers and loss or disruption of the cortical actin band (110, 140, 180, 255). We further observed that exposure of bovine microvascular endothelial cells to a period of hypoxia followed by reoxygenation induces the production of sufficient amounts of O2− to increase actin stress fiber formation and increase endothelial permeability (144). Using a replication-deficient adenovirus vector, Crawford et al. (42) overexpressed superoxide dismutase in human aortic endothelial cells and found that the reoxygenation-induced actin reorganization was inhibited. These findings provide evidence that the actin cytoskeleton is a target of oxidant stress action.

One mechanism of oxidant-mediated regulation of actin is through the actin-binding proteins. Filamin is a nonmuscle cross-linking actin-binding protein that connects actin microfilaments to membrane glycoproteins and appears to be sensitive to oxidant stress. Hastie et al. (86) observed that in human umbilical vein endothelial cells treated with H2O2, filamin rapidly translocated from the membrane to the cytosol, followed by changes of actin microfilament reorganization and intercellular gap formation, a sequence of events suggestive of a possible causal relation between filamin and barrier impairment. Hastie et al. (88) observed that H2O2-activated generation of phospholipase D (PLD) metabolites may mediate filamin redistribution and actin filament reorganization. There is also evidence that ROS such as H2O2 can directly oxidize actin thiols (in particular, the sulphhydryl group of Cys-374), which likely alters the actin carboxy terminus, leading to impairment of actin subunit interactions as well as binding with actin-binding proteins (43).

Another characteristic of oxidant stress in endothelial cells is a rapid fall in cellular ATP levels (92, 218), resulting from inactivation of the glycolytic and mitochondrial pathways of ADP phosphorylation (203). The oxidant-mediated decreased ATP level is accompanied by actin microfilament disruption (92) and increased endothelial permeability (97). The F-actin microfilament disruption can be mimicked by decreasing the ATP level with glucose depletion and metabolic inhibition (92, 127). The metabolically induced ATP depletion also is accompanied by increased permeability (29, 151, 188). Additionally, metabolic inhibition potentiated the oxidant-mediated reorganization of F-actin microfilaments in endothelial cells (44). Although the precise relationship of decreased ATP with F-actin reorganization and increased permeability is unclear, these observations indicate that decreased ATP may be a critical component of oxidant stress-induced barrier dysfunction.

OXIDANT-MEDIATED SIGNALING MECHANISMS IN ENDOTHELIAL BARRIER FUNCTION

There is convincing evidence that low subcytotoxic concentrations of ROS function as important physiological signaling molecules mediating basic cellular activities such as cell growth and differentiation (Fig. 3) (112, 164). ROS are ubiquitous and short lived, and they likely regulate signaling cascades at multiple points. For example, the phospholipases PLA2, PLC, and PLD are activated by ROS, which in turn generate
a host of cellular messengers and cofactors that regulate further downstream cellular activities, including protein kinases and phosphatases (164). Additionally, ROS may directly modulate activity of the downstream molecules. There is also evidence suggesting that sublethal levels of oxidant stress inactivate tyrosine and serine/threonine protein phosphatases, contributing to increased activities of protein kinases in several signaling pathways (240). The cellular targets modulated by ROS are highly dependent on the lipid solubility, cellular site of oxidant generation, and half-life of the particular oxidant. We next present some emerging data regarding how ROS modulate several key signaling pathways that are implicated in the regulation of permeability and endothelial cell-leukocyte interactions. Overall, these reports suggest likely complex multiple regulatory mechanisms that control endothelial barrier function under oxidant stress conditions. Further research is needed at the molecular level to delineate the precise role and contribution of the signal transduction systems involved.

**Altered Intracellular Ca^{2+} Regulation**

Abundant evidence indicates that oxidant stress of the endothelium increases the intracellular calcium concentration ([Ca^{2+}]_i) (46, 214, 234), which correlates with increased endothelial permeability (208, 214). Direct treatment of endothelial cells with H_2O_2 increased [Ca^{2+}]_i, within minutes, and the increase appeared to be sustained (214, 234). The chelation of extracellular Ca^{2+} (214) as well as treatment with the PLC inhibitor U-73122 (234) inhibited in part the oxidant-induced increased [Ca^{2+}]_i, suggesting that Ca^{2+} influx and mobilization, respectively, contributed to the overall increased [Ca^{2+}]_i.

The increased influx mediated by xanthine/xanthine oxidase has been recently reported to be inhibited by an anion channel blocker, Ni^{2+} (an inorganic membrane Ca^{2+} channel blocker), dithiothreitol, and inhibitors of the Haber-Weiss reaction (15). These observations suggest that Ca^{2+} influx occurred through membrane Ca^{2+} channels that were regulated by zOH generation. The mechanism of mobilization of Ca^{2+} from intracellular stores is likely through activation of PLC. The treatment of pulmonary artery endothelial cells with H_2O_2 results in the hydrolysis of PIP_2, producing diglycerides, phosphatidic acid, and inositol phosphates (48, 211). The generated inositol 1,4,5-trisphosphate (IP_3) is a known ligand of the IP_3-activated Ca^{2+} channel on endoplasmic reticulum for Ca^{2+} mobilization (55). However, both peroxide (78) and zOH (134) also have been shown to inhibit the endoplasmic reticular Ca^{2+}-ATPase, causing increased [Ca^{2+}]_i. Normally, Ca^{2+}-ATPases function to lower [Ca^{2+}]_i by transporting Ca^{2+} from the cytosol into endoplasmic reticulum. There is some evidence that the inhibition was the result of the zOH modifying key sulfhydryl groups on the Ca^{2+}-ATPase molecule (134).

The inorganic Ca^{2+} entry blocker LaCl_3 inhibited the increase in albumin permeability of porcine pulmonary artery endothelial cells in response to ROS generated by xanthine/xanthine oxidase (208), yet it was ineffective in inhibition of the H_2O_2-mediated increased permeability of bovine pulmonary microvascular endothelial cells (214). These contrasting findings may be attributable to the species difference of cells. Alterna-
tively, they may be related to different regulatory mechanisms of permeability utilized by endothelial cells from conduit vs. microvascular vessels. Endothelial cells from different tissue sites are known to be distinct in their nutritional requirements and responses to growth and migration stimuli (254), antioxidant profiles (231), surface expression of glycoproteins (22), and Ca\(^{2+}\) regulation (37, 115). These observations point to our incomplete understanding regarding mechanisms by which the rise in [Ca\(^{2+}\)]\(_i\) regulates endothelial permeability.

Although increased [Ca\(^{2+}\)]\(_i\) has been correlated with endothelial barrier dysfunction in response to ROS as well as other edemagenic agents (38, 67, 89, 115, 145, 198, 214, 223), the underlying cellular and molecular basis remains to be resolved. The current understanding is that the increased permeability consists of Ca\(^{2+}\)-dependent and -independent components, and an increased [Ca\(^{2+}\)]\(_i\), alone is likely not sufficient to fully promote increases in permeability. The Ca\(^{2+}\)-dependent component of the permeability response may involve regulation of myosin light chain kinase (MLCK) (66, 212), the classic PKC isoforms (28, 235), and the Ca\(^{2+}\)-inhibitable adenylyl cyclase (221).

**Myosin Light Chain Kinase**

Recent findings indicate that H\(_2\)O\(_2\) treatment of endothelial cells increases MLC phosphorylation (142, 255), suggesting that endothelial contraction plays an important role in the oxidant stress-induced endothelial barrier dysfunction. One important basis of increased vascular endothelial permeability is the activation of endothelial contraction. Permeability-increasing mediators such as thrombin and histamine also increase MLC phosphorylation (66, 147, 163), which precedes the onset of endothelial contraction. Furthermore, maximal phosphorylation has been maintained during sustained contraction (73). Activated MLCK directly phosphorylates Thr-18 and Ser-19 of MLC, which are related to isometric tension development and increased actin polymerization in endothelial cells (73). Inhibition of MLCK with ML-7, which competes with ATP for binding to the kinase, reduces both the thrombin- and histamine-induced increased MLC phosphorylation and endothelial permeability (66, 163). These findings support the thesis that actin-myosin contraction increases centripetally directed tension that opposes and overcomes the centrifugal tethering forces, resulting in a decrease or loss of cell-cell adhesion and the subsequent barrier dysfunction. Additionally, inflammatory mediators may inactivate myosin-associated protein phosphatases, further promoting MLC phosphorylation (53, 210). ROS have been reported to inactivate serine/threonine protein phosphatases (240). However, increasing evidence indicates that increased endothelial permeability can also be independent of increased MLC phosphorylation (67, 163, 178, 207), implicating the involvement of other regulatory mechanisms such as PKC, tyrosine kinases, and Rho GTPase proteins.

**Protein Kinase C**

**Evidence for PKC in oxidant-mediated barrier dysfunction.** Several lines of evidence implicate the involvement of PKC in mediating the increased vascular endothelial permeability in response to oxidant stress. In guinea pig lungs pretreated with H-7 (a PKC inhibitor acting on the catalytic site of the enzyme), the increase in pulmonary capillary filtration coefficient in response to perfusion of H\(_2\)O\(_2\) was inhibited (110). In cultured monolayers of pulmonary microvascular endothelial cells, H-7 or calphostin C (which inhibits the regulatory site of the enzyme) prevented the H\(_2\)O\(_2\)-induced increase in albumin permeability (213). Furthermore, the increases in permeability were accompanied by reorganization of actin cytoskeleton, which was also inhibited by the PKC inhibitors (110, 213). In another study, PKC inhibitors blocked both the H\(_2\)O\(_2\)-induced increases in endothelial permeability as well as increased MLC phosphorylation (142).

**Oxidants activate PKC.** ROS activate PKC by at least three mechanisms. One mechanism is a direct oxidative modification of the regulatory domain, resulting in increased PKC activity independent of Ca\(^{2+}\) and phospholipids (74). However, the specific site(s) of oxidative modification of the enzyme has yet to be identified. A second mechanism by which ROS activate PKC is through mediators and cofactors generated secondary to activation of PLC, PLD, and PLA\(_2\). In endothelial cells, ROS cause activation of PLC and PLA\(_2\) (35, 48, 211) as well as PLD (165), leading to production of potent activators of PKC [i.e., Ca\(^{2+}\), diacylglycerol (DAG), free fatty acids, and phosphoinositides] (139, 164, 171, 224). Finally, oxidant-mediated activation of PKC also may occur through phosphorylation of distinct residues of different PKC isoforms. The treatment of COS-7 cells, which had been transfected with individual PKC isoforms, with H\(_2\)O\(_2\) resulted in tyrosine phosphorylation and catalytic activation of the classic PKC (cPKC) isoforms α, β, and γ; the novel PKC (nPKC) isoforms δ and ε; and the atypical PKC (aPKC) isoform ζ (126). Further evaluation of PKC-δ showed that the phosphorylation was restricted to tyrosine residues, particularly Tyr-512 and Tyr-523, in the carboxy terminal half of the enzyme that are critical for PKC activation (126). The upstream events responsible for the tyrosine phosphorylation of PKC isoforms are unknown. ROS are known to activate several members of the Src family of protein tyrosine kinases (PTK) in a wide range of cell types, including endothelial cells (3, 16, 84, 183, 202). The PTK implicated in regulation of PKC and endothelial barrier function remain to be elucidated.

**Mechanisms of PKC in regulation of barrier function.** PKC is a family of serine/threonine protein kinases consisting of at least 12 known isoforms, which are classified primarily by cofactor requirements for activation and function (122, 139, 171). The cPKC isoforms α, βI, βII, and γ are activated by Ca\(^{2+}\), phosphatidylyserine, and DAG, whereas the nPKC isoforms δ, ε, θ, and η are activated by phosphatidylserine and DAG.
and do not require Ca\(^{2+}\). The aPKC isoforms \(\xi, \lambda, \) and \(\iota\) are activated by phosphatidylinositol but do not require DAG or Ca\(^{2+}\). With some isoforms, cis-unsaturated free fatty acids (e.g., arachidonic, linoleic, and oleic acid) generated by PL\(_{A2}\)-mediated hydrolysis of phospholipids enhance the DAG-dependent activation of the isoforms (139, 171). These isoforms are distributed to different intracellular sites, are selectively sensitive to PKC inhibitors and downregulation, and have different selectivity for substrates, suggesting that the isoforms have unique functions (139). In endothelial cells, PKC-\(\alpha\) and -\(\beta\) isoforms are the primary Ca\(^{2+}\)-dependent isoforms, whereas the novel isoforms \(\delta\) and \(\epsilon\) as well as the atypical isoforms \(\zeta\) and \(\lambda\) comprise the predominant Ca\(^{2+}\)-independent isoforms (28, 83, 235, 237, 256).

The endothelial adherens and tight junctions and associated proteins can exist as phosphoproteins (41, 72, 189, 219, 233), suggesting that their function may be regulated by the phosphorylation state of the proteins. Phosphorylation by PKC is a well-documented mechanism for mediator-induced increases in permeability, although the precise molecular events remain undefined. Pretreatment of cultured endothelial cell monolayers with inhibitors of PKC (i.e., H-7 and staurosporine) attenuates the increased albumin permeability in response to thrombin (149, 220). Furthermore, the PKC inhibitor calphostin C has been shown to prevent thrombin-induced loss of \(\gamma\)-catenin, \(\alpha\)-catenin, and \(\beta\)-catenin, and p120 proteins of the cadherin immunocomplex (191), suggesting that PKC-mediated phosphorylation regulates shifts in intracellular pools of cadherin/catelin. Current evidence indicates that increases in endothelial permeability are regulated by the classic PKC isoforms. Expression of full-length antisense PKC-\(\beta\) cDNA into endothelial cells had been shown to reduce the protein levels of endogenous PKC-\(\beta\), accompanied by inhibition of the phorbol 12-myristate 13-acetate-mediated increase in endothelial permeability (235). However, thrombin-induced increases in permeability as well as [Ca\(^{2+}\)]\(_i\), have been potentiaged by expression of antisense PKC-\(\beta\) (235), suggesting that PKC-\(\beta\) may downregulate signaling pathways activated by the thrombin receptor. Recently, the TNF-\(\alpha\)-mediated increase in endothelial permeability was correlated to a prolonged activation pool of PKC-\(\alpha\) (56). The roles of other PKC isoforms in endothelial barrier function regulation remain to be determined.

**Mitogen-Activated Protein Kinase**

The mitogen-activated protein (MAP) kinase pathway can be activated by reactive oxidant stimuli such as H\(_2\)O\(_2\) or ischemia-reperfusion and is a key cellular response to oxidant stress (2, 82, 238). The upstream regulator of MAP kinase, p21\(^{ras}\), has been shown to be a direct target of ROS and is believed to be a central mechanism by which redox stress signals are transmitted (133). MAP kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a variety of proliferation and stress stimuli (113). The MAP kinase family consists of three distinct groups, the extracellular signal-regulated kinase (ERK), the c-Jun amino-terminal kinase (JNK), and the p38 MAP kinase. The ERK pathway is implicated in the endothelial barrier dysfunction caused by phorbol esters, functioning downstream of Ras activation (232).

The involvement of MAP kinase in oxidant-induced endothelial barrier dysfunction has been little studied, although the p38 kinase pathway is known to contribute to actin filament reorganization in response to oxidant stress (79, 104, 105). Recent work by Kevil and coworkers (119) showed that the H\(_2\)O\(_2\)-mediated endothelial increase in permeability was accompanied by ERK1/2 phosphorylation, occludin redistribution, and phosphorylation. Furthermore, these H\(_2\)O\(_2\)-mediated activities were inhibited by PD-98059, an ERK kinase (MEK) inhibitor.

**Tyrosine Kinases**

Increasing evidence indicates that the PTK pathway is important in mediating endothelial barrier dysfunction. The promotion of phototyrosine content by treatment of endothelial cells with tyrosine phosphatase inhibitors (i.e., phenyl arsine oxide and sodium orthovanadate) increases solute permeability (236, 253). Several edemagenic mediators may increase endothelial permeability through tyrosine kinase-mediated phosphorylation of junctional proteins. VEGF treatment (15–90 min) of endothelial cells has resulted in tyrosine phosphorylation of VE-cadherin, \(\beta\)-catenin, plakoglobin (\(\gamma\)-catenin), p120, and platelet endothelial cell adhesion molecule-1 (PECAM-1) from human umbilical vein endothelial cells (52) and occludin-1 and ZO-1 from bovine retinal endothelial cells (10). Although the functional significance of tyrosine phosphorylation is not clear, this may provide a means to target the junctional proteins for degradation (236). Furthermore, tyrosine residues of paxillin and focal adhesion kinase (FAK) have been phosphorylated in bovine coronary venular endothelial cells stimulated with histamine or phorbol esters; the tyrosine kinase inhibitor damnacanthal abolished this phosphorylation as well as the increased permeability (253).

Endothelial cells stimulated with H\(_2\)O\(_2\) showed increased tyrosine phosphorylation of proteins at focal contacts (77, 230). Interestingly, inhibition of tyrosine phosphorylation with genistein prevented H\(_2\)O\(_2\)-mediated increases in tyrosine phosphorylation, intercellular gap formation, and monolayer permeability (30), implicating the involvement of PTKs in the oxidant-mediated permeability response.

**Rho GTPases**

The family of Rho GTPases are essential in the relay of signals to the actin cytoskeleton involved in a multitude of cell activities such as cell adhesion, motility, polarity, cell cycle progression, and apoptosis. The family consists of 14 distinct members (Rac1–3, Cdc42,
TC10, RhoA–E, G, and H, and Rnd1 and 2), of which Cdc42, Rac1, and RhoA are well characterized (13). Studies in fibroblasts indicate that RhoA causes stress fiber and focal contact formation, Cdc42 regulates filopodia formation, and Rac1 regulates the formation of lamellipodia.

RhoA promotes MLC phosphorylation in endothelial cells as in other cell types, suggesting a possible mechanism by which Rho GTPases regulate barrier function (8, 53, 65, 121). However, the involvement of Rho GTPases in the regulation of endothelial barrier function remains unclear and controversial. In some studies in which RhoA was inactivated in endothelial cells, the thrombin-mediated barrier dysfunction was reduced (9, 53), whereas in another study there was no inhibition (31). Rho GTPases may be crucial for the regulation and function of adherens junctions in endothelial cells (24, 135). Indeed, the expression of mutant forms of RhoA and Rac was shown to inhibit the formation of junctions in endothelial cells (135). Although there is little information regarding the effects of ROS on Rho GTPase function, ROS are known to dramatically remodel the endothelial actin cytoskeleton, suggesting that the Rho GTPases may mediate these actin responses. Further studies are needed to understand fully the mechanisms by which this important modulator of the cell cytoskeleton regulates endothelial barrier function under oxidant stress conditions.

**LEUKOCYTE EXTRAVASATION AND OXIDANT STRESS**

As mentioned earlier (EFFECTS OF OXIDANT STRESS ON VASCULAR ENDOTHELIUM), numerous experimental models have documented increased leukocyte extravasation in response to oxidant stress (100, 190, 200). Furthermore, using intravital microscopy, Gaboury et al. (63) showed that infusion of hypoxanthine/xanthine oxidase into the rat mesenteric circulation increased the number of both rolling and adherent leukocytes.

**Current Understanding of Leukocyte Extravasation**

It is now recognized that the endothelium plays a key role in the control of leukocyte adhesion and trafficking. Leukocyte extravasation, a vital host-defense response, consists of precisely coordinated series of interactions between leukocytes and endothelial cells that direct leukocytes out from the circulation to extravascular tissue sites. At sites of inflammation, infection, or injury, local mediators activate circulating leukocytes and/or the endothelium, whereby the leukocytes bind to endothelial cells with low-affinity intermittent adhesions (rolling) on the endothelial surface. These initial events are believed to be mediated by the selectin family of adhesion molecules (L-selectin on leukocytes, P- and E-selectins on endothelial cells) that initiate rolling of leukocytes on the endothelial surface (Fig. 4) (157). In endothelial cells, P-selectin is stored within the Weibel-Palade bodies and can be rapidly recruited to the cell surface upon activation, whereas E-selectin expression requires de novo synthesis by activation of the E-selectin gene. The initial adhesion is followed by development of a more firm adhesion between leukocytes and endothelial cells, which is postulated to be required for subsequent transmigration. The firm adhesion is primarily mediated by activation of the β2-integrin CD18 (i.e., of PMN and macrophages) or the β1-integrin CD29 (i.e., of lymphocytes) and/or upregulation (functional or quantitative) of the endothelial counter receptors, the immunoglobulin (IgG) supergene family of adhesion molecules including intercellular adhesion molecule (ICAM)-1 and ICAM-2 and vascular cell adhesion molecule (VCAM)-1 (216). The firm adhesion of leukocytes onto the vascular endothelial surface is regarded to be an essential step leading to transmigration through the intercellular junctions (diapedesis). Thus successful recruitment of leukocytes to sites of host defense requires a concerted series of complex interactions between the leukocyte and endothelium. However, under pathological condi-
tions, in which localized stimuli such as cytokines cause excessive and prolonged leukocyte and endothelial cell activation responses, excessive extravasation may occur, resulting in tissue damage (136). During acute inflammation, PMN are mobilized within minutes to hours upon stimulation, whereas monocytes are localized to sites of infection within approximately a day. Although the general principals of leukocyte trafficking are similar, it is becoming increasingly apparent that the underlying cellular and molecular mechanisms are highly dependent on the inflammatory stimulus, the specific leukocyte population, and the tissue involved.

**Basis for Endothelial-Leukocytic Firm Adhesion**

Oxidants promote leukocyte adhesion to endothelium. There is clear indication that oxidant stress promotes increased leukocyte adhesion to vascular endothelium (63, 141, 176). This increased leukocyte adhesion has been demonstrated to be attributable to ICAM-1 and selectin-dependent adhesion mechanisms in several experimental systems. In the rat mesenteric circulation, increased rolling and adherent PMN occurred in response to hypoxanthine/xanthine oxidase infusion and were inhibited by antibodies directed against CD18 or P-selectin (63). In human umbilical vein endothelial cells, treatment with H$_2$O$_2$ increased expression of P-selectin within 1 h and was sustained for up to 3 h, and antibody to P-selectin abolished PMN adhesion to the endothelial cells (176). Similarly, oxidant-mediated increased PMN adhesion to endothelium was associated with increased expression of ICAM-1 (141), and antibody to ICAM-1 inhibited PMN adhesion (141, 206). This upregulation of ICAM-1 has been shown to be associated with the activation of transcription through AP1/Ets elements within the promoter (195, 196). Furthermore, oxidant stress may induce multiple phases of response by adhesion molecules. Oxidant stress produced by a redox imbalance (i.e., reduced glutathione) has been shown to cause a biphasic increase in PMN adhesion to human umbilical vein endothelial cells, with peak responses at 15 and 240 min, both inhibited by antibodies to P-selectin or ICAM-1 and correlated to increased surface expression of the adhesion molecules (124). Overall, these results suggest that leukocyte adhesion is mediated by existing adhesion molecules on the endothelium and by de novo synthesis (Fig. 1). In contrast, others (29) reported a lack of transcriptional activation and expression of ICAM-1 in response to H$_2$O$_2$, suggesting an absence of de novo synthesis of ICAM-1 or that ICAM-1-independent adhesion pathways exist (see below). These different findings underscore the incomplete understanding of the molecular basis of leukocyte adhesion and transmigration under oxidant stress conditions.

Recent studies stress two factors important in the understanding and interpretation of cellular mechanisms of leukocyte extravasation. Nishio and coworkers (170) reported that in hyperoxia-exposed rat lungs, P-selectin was sparsely expressed in arterioles, whereas ICAM-1 was expressed significantly in venules and capillaries, which was associated with firm adhesion in the capillaries but not in arterioles or venules. These findings indicate regional differences in both expression and function of endothelial surface adhesion molecules, suggesting possible regional susceptibility to oxidative stress and/or intrinsic differences in expression. Furthermore, oxidant-responsive transcription factors are regulated in a cell type-specific manner. We recently demonstrated that oxidant induction of ICAM-1 and the PMN chemoattractant IL-8 is cell-type dependent and associated with differential activation and binding of activator protein-1 (AP-1) and nuclear factor (NF)-κB (130, 131, 192). H$_2$O$_2$ selectively induced ICAM-1 expression in endothelial cells and IL-8 in epithelial cells (130, 193). H$_2$O$_2$ induction of IL-8 mRNA in epithelial cells was concentration dependent and corresponded to H$_2$O$_2$ induction of AP-1 binding to the IL-8 promoter (131). Thus ROS, in contrast to cytokines, activate IL-8 and ICAM-1 gene expression in a cell type-specific manner, and oxidant signaling appears to be an important mechanism for differential gene expression in endothelial and epithelial cells. These studies point to the important notion that regulation of leukocyte adhesion and transmigration is governed by intrinsic factors that differ among regions within the tissue, different tissues, and cell types.

**Regulation of ICAM-1 function.** Despite the current contrasting findings regarding the regulation of ICAM-1 expression by ROS, it is generally agreed that the most important adhesion molecules in mediating firm adhesion of leukocytes to the endothelial surface after the initial rolling events are members of the IgG family (i.e., ICAM-1, ICAM-2, VCAM-1). Both ICAM-1 and ICAM-2 are constitutively expressed on endothelial cells, but only ICAM-1 can be upregulated transcriptionally (195) (Fig. 1, Table 2). Intracellular vesicular pools of ICAM-1 have been detected in synovial endothelial cells, in which cytokines can induce the release and surface expression of the stored ICAM-1 (33). Functional upregulation of the ICAMs can also occur through changes in conformation and/or distribution of the constitutively expressed forms, resulting in increased affinity (206). Evidence indicates that ICAM-1 is anchored to the actin cytoskeleton through association with α-actinin (20, 34). Both ICAM-1 and ICAM-2 are found to associate with ezrin, a protein known to link the actin cytoskeleton to the cell membrane (90). The Rho GTPases, central in regulation of actin organization and function, have been implicated in the function of these adhesion molecules. In a study by Wójciak-Stothard et al. (243), the inhibition of RhoA activity with C3 transferase or N19RhoA (dominant-negative RhoA) resulted in inhibition of clustering of E-selectin, ICAM-1, and VCAM-1 and reduction of adhesion of monocytes to activated endothelium. These findings support a mechanism for stable adhesions between the endothelium and leukocytes in which the actin filament network and its associated regulatory molecules function to redistribute/assemble clustering
of adhesion molecules. The specific role(s) of endothelial actin filaments and Rho GTPases in the regulation of leukocyte adhesion and transmigration by ROS remain to be shown.

**ICAM-1-independent adhesion.** It is increasingly apparent that ICAM-1-independent adhesion pathways exist (26, 57, 150, 161, 245). For example, double-knockout mice deficient in P-selectin and ICAM-1 showed a complete lack of PMN emigration into the peritoneum during *Streptococcus pneumoniae*-induced peritonitis, whereas PMN emigration was not inhibited in *S. pneumoniae*-induced pneumonia, indicating an ICAM-1-independent response in the lung (26). However, in pneumonia induced with either *Escherichia coli* lipopolysaccharide or *Pseudomonas aeruginosa*, CD18-deficient PMN, but not wild-type PMN, showed impairment in emigration, whereas in pneumonia induced with *S. pneumoniae*, emigration of CD18-deficient and wild-type PMN was similar, indicating stimulus specificity of the responses within the same tissue (161). This latter study is further supported by a recent in vitro study in which PMN transmigration was determined in response to three chemoattractants: fMLP, leukotriene B4 (LTB4), or IL-8 (150). These authors reported that function-blocking antibody to CD18 decreased ~70% of fMLP-mediated and ~20% of LTB4-mediated PMN transmigration but had no effects on IL-8-mediated transmigration. Interestingly, the study also showed that both the LTB4- and IL-8-mediated PMN transmigrations were insensitive to blockade with antibodies to P-selectin, E-selectin, and CD29. These findings indicate that activation of ICAM-1-mediated adhesion in endothelial cells is highly dependent on the inflammatory stimulus and that ICAM-1-independent pathways exist for leukocyte adhesion and transmigration. It is not known whether oxidant stress of endothelial cells may also mobilize ICAM-1-independent adhesion pathways.

**Redox-Sensitive Transcription Factors**

The final targets of signal transduction pathways are nuclear transcription factors. AP-1 and NF-κB are two major redox-sensitive transcription factors that are activated through the PKC and MAP kinase pathways and mediate endothelial cell gene responses to oxidant stress (Fig. 5). AP-1 is a large family of basic leucine zipper (bZip) transcription factors that are induced by growth factors, tumor-promoting phorbol esters, and regulatory cytokines [reviewed by Karin et al. (114)]. AP-1 consists of two subfamilies, Jun (members include c-Jun, JunB, and JunD) and Fos (members include c-Fos, FosB, Fra1, and Fra2), that bind a common DNA recognition site as either Jun homodimers or as more stable Jun/Fos heterodimers. Jun and Fos can also form heterodimers with the cAMP response element binding protein (CREB) transcription factor family (185, 194).

AP-1 binding activity in vitro is regulated by the redox status of a single conserved cysteine residue in the DNA binding domains of Jun and Fos (1, 169). This cysteine must be in the reduced state for DNA binding to occur, and changing it to a serine residue results in an increase in AP-1 binding activity that is no longer redox regulated (174). An AP-1 regulatory protein, called Ref-1, has been identified and shown to regulate AP-1 binding activity in vivo through its redox effects (250, 251). Ref-1 activity itself is regulated by a redox mechanism involving thioredoxin and by phosphorylation by casein kinase II (58, 94).

The three MAP kinase pathways regulate AP-1 activity both by increasing the transcription of the *jun* and *fos* genes and by phosphorylation of newly synthesized AP-1 transcription complexes. c-Fos gene transcription is activated by the cooperative interaction of Elk-1 with the serum response factor (SRF). Phosphorylation of Elk-1 by ERK, JNK, and p38 MAP kinases increases ternary complex formation with the SRF, thereby activating c-Fos gene transcription. The activation of c-Jun gene transcription is mediated by two AP-1 binding sites within the *jun* promoter that are constitutively occupied by heterodimers of c-Jun and ATF-2. JNK and p38 kinases phosphorylate c-Jun and ATF-2 on specific serine residues that are required to activate transcription of the *jun* gene (40). Newly synthesized AP-1 is also regulated by the MAP kinases. JNK phosphorylates c-Jun, whereas ATF-2 is phosphorylated by both JNK and p38.

The NF-κB family of transcription factors is composed of two groups of structurally related, interacting proteins that bind κB elements as dimers and whose activity is regulated by subcellular location (reviewed in Ref. 19). NF-κB family members of the first group

<table>
<thead>
<tr>
<th>Adhesion Molecule</th>
<th>Synonym</th>
<th>Constitutive (C), Inducible (I) Major Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-selectin</td>
<td>CD62P</td>
<td>C, I PSGL-1</td>
</tr>
<tr>
<td>E-selectin</td>
<td>CD62E</td>
<td>I PSGL-1, ESL-1</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CD54</td>
<td>C, I LFA-1 (CD11a/CD18); Mac-1 (CD11b/CD18); gp150/95 (CD11c/CD18)</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>CD102</td>
<td>C LFA-1; Mac-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>CD106</td>
<td>C, I α4/β7 (CD29, VLA-4); αv/β3</td>
</tr>
</tbody>
</table>

**Expression on Luminal Surface**

| Immunoglobulins   |                  |                                                     |
| PECAM-1           | CD31             | C CD31, α4/β7                                       |
| Junctional proteins | Cadherin         | C Cadherin, integrin                                |
|                   | Ocludin          | C Ocludin                                           |

| Immunoglobulins   |                  |                                                     |
| PECAM-1, P-selectin glycoprotein; ESL-1, E-selectin ligand; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule. |

**Expression on Lateral Membrane**

**Table 2. Endothelial adhesion molecules in leukocyte trafficking**
include NF-κB1 (p50) and NF-κB2 (p52), which are synthesized as precursor proteins. The second group includes RelA (p65), RelB, and c-Rel, which are synthesized as mature proteins containing one or more potent transactivation domains. Each NF-κB subunit has distinct binding and transactivation properties, and when combined as homo- or heterodimers, they form transcriptional activators with distinct activities (129). In resting cells, latent NF-κB is complexed to a class of cytoplasmic retention proteins called inhibitors of NF-κB (IkB). Signals that induce NF-κB activity result in the phosphorylation of IkB on specific serine residues, marking the protein inhibitor for ubiquitination and subsequent proteolytic degradation by the ATP-dependent 26S proteasome complex. The newly released NF-κB is then free to move to the nucleus, bind to its recognition site, and activate gene transcription.

The NF-κB signaling pathway is activated by the proinflammatory cytokines TNF-α and IL-1α, which are the major cytokine inducers of gene expression in endothelial cells. These cytokines increase intracellular ROS, and their effects can be suppressed by thiol antioxidants such as N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) (186, 196). TNF-α activates NF-κB through a kinase-mediated phosphorylation cascade involving a high-molecular-mass kinase complex (159). Cytokine activation of the multi-protein IkB kinase complex, which contains two kinase components, the IkB kinase (IKK)/heterodimer and NF-κB-inducing kinase (NIK), results in the serine phosphorylation of IkB and the subsequent activation of NF-κB (249). Blockage of IkB phosphorylation by serine proteases or IkB degradation by inhibition of the 26S proteasome has been shown to suppress the TNF-α induction of gene expression (36, 109).

In addition to phosphorylation events, intracellular thiol redox status appears to be a critical determinant of NF-κB activation. The major intracellular thiol, the triamino acid peptide glutathione (GSH), inhibits the serine phosphorylation of IkB, which is required for NF-κB activation (39). NAC, a precursor of GSH, also has been shown to inhibit NF-κB activation (204). Thus, at high levels of cytosolic GSH, NF-κB is not activated. However, NF-κB binding activity is also inhibited by high concentrations of glutathione disulfide (GSSG), the oxidized form of GSH, suggesting that optimal cellular redox ratios of GSH to GSSG are required for effective NF-κB activation (47, 64) and possibly explaining the cell type-specific redox regulation of NF-κB. Indeed, GSH-depleting agents such as diamide and buthionine sulfoximine (BSO) alter the intracellular redox state and inhibit oxidant-induced activation of NF-κB as well as AP-1 (i.e., c-Jun/ATF-2) (124, 197).

Recently, it was demonstrated that modulation of the GSH/GSSG ratio in endothelial cells produces a biphasic PMN-endothelial cell adhesion response mediated by transcription-dependent and -independent surface expression of different endothelial cell adhesion molecules (124).
Another physiologically important thiol that contributes to the regulation of NF-κB activity is thioredoxin, an oxidoreductase with potent antioxidant properties (201). Thioredoxin increases NF-κB binding activity through the reduction of a redox-sensitive cysteine residue in the DNA binding domain (128, 160). The cysteine residue must be maintained in a reduced state to allow DNA binding. Paradoxically, antioxidants such as α-lipoate, NAC, and PDTC inhibit oxidant-mediated NF-κB activation induced by TNF-α, phorbol ester, or H₂O₂ (204), suggesting that the redox regulation of NF-κB, like that of AP-1, is complex and mediated by both oxidant and antioxidant mechanisms.

Recently, in addition to AP-1 and NF-κB, oxidant stress has been shown to modulate the activity of the transcription factor STAT (signal transducers and activators of transcription), a family of transcription factors activated by tyrosine phosphorylation signals transmitted from cytokine receptors such as interferons and interleukins (Fig. 5). Receptor activation initiates via receptor-associated kinases the phosphorylation of specific tyrosine residues within the STAT transactivation domain, which allows for the dimerization and nuclear translocation of STAT transcription factors. Nuclear localization of STAT allows the dimeric factors to interact in a sequence-specific manner with the IFN-γ response sequence (GAS) and the interferon-stimulated response element (IRE). The binding of STAT to these promoter elements activates transcription of cytokine responsive genes such as ICAM-1 in endothelial cells.

The reactive oxidant H₂O₂ activates the STAT signaling pathway, promoting the nuclear translocation and binding of STAT-1 and STAT-3 to their respective promoter elements (32, 215). Also, H₂O₂ stimulates the activity of the known STAT kinases JAK2 and TYK2. Moreover, intracellular oxidant stress activates the STAT pathway, because oxidized low-density lipoprotein or depletion of intracellular glutathione enhances STAT-1 and STAT-3 binding activity (156).

Transmigration Through Endothelial Junctions

Relation to barrier dysfunction. The increased endothelial permeability caused by ROS may facilitate leukocyte transmigration (117, 119). Most current evidence indicates that leukocyte extravasation involves migration of the leukocyte between endothelial cells (i.e., through intercellular junctions). Studies of the relationship between leukocyte diapedesis and endothelial barrier function suggest that these events are associated with some degree of barrier impairment (70, 227). In one study, the addition of PMN (at a PMN-to-endothelial cell ratio of 10:1) in response to fMLP or LTB₄ caused a rapid (within minutes) transendothelial resistance drop that preceded PMN transmigration (70), suggesting that barrier impairment may lead to transmigration. In support of this notion are studies demonstrating that antibody to VE-cadherin increases endothelial permeability as well as PMN transmigration (75, 98). In contrast, in another study, no change in transendothelial resistance was detected for up to 30 min after addition of PMN (using the same PMN-to-endothelial cell ratio as reported above) in response to fMLP (101). Furthermore, in a study in which albumin flux and transendothelial migrations of lymphocytes were monitored continuously, increased transmigration occurred after the increased albumin clearance rate had returned toward baseline (49). Therefore, the notion that leukocyte transmigration requires increased endothelial permeability remains somewhat controversial.

Interestingly, several studies have demonstrated that leukocyte adhesion and transmigration remodel endothelial junctions. In an immunofluorescence study, adhesion of bovine coronary venular endothelial cells with C5a-activated PMN resulted in loss of VE-cadherin and β-catenin, increased stress fibers, and intercellular gap formation (227). In this same study, (227) phosphorylation of VE-cadherin and β-catenin was accompanied by endothelial permeability. Moreover, adhesion of PMN to TNF-α-activated human umbilical vein endothelial cells also resulted in loss of VE-cadherin and its associated proteins as detected by immunofluorescence (6, 45). Corresponding immunoprecipitation studies corroborated this loss of adherens junctional proteins; however, there was a concern that these latter findings were caused by PMN proteases released upon detergent lysis of cells (162). A more recent immunofluorescence confocal microscopic study by Allport and coworkers (7) found that under flow conditions, monocyte transmigration induces focal reversible loss of VE-cadherin and β-catenin. The authors hypothesized that the VE-cadherin complex was disrupted locally only by active transmigration and that the complex resealed after diapedesis. The finding of reversible junctional remodeling bolsters the argument that loss of adherens junctional proteins accompanied the leukocyte adhesion and transmigration events.

PECAM-1 is another adhesion molecule important for leukocyte extravasation and has been implicated in the regulation of leukocyte transmigration in oxidant stress. PECAM-1 is located at the endothelial lateral cell border and also is expressed by leukocytes; thus it is able to form homotypic as well as heterotypic adhesions. Superfusion of H₂O₂ onto rat mesenteric microvascular results in increased leukocyte extravasation inhibitable by antibody to PECAM-1 (200). Similarly, antibody to PECAM-1 inhibits the increased transmigration of monocyte-like HL-60 cells across endothelial cells activated by the oxidant tert-butyldihydroperoxide (190).

Endothelial signaling events involved in transmigration. The oxidant-mediated increased [Ca²⁺], as well as phosphorylation of MLC in endothelial cells may provide critical regulatory signals in the regulation of leukocyte transmigration. Transient increases in endothelial [Ca²⁺] occur in response to PMN adhesion to and transmigration across fMLP- or IL-1-activated human umbilical vein endothelial cells (102). Abrogation of the [Ca²⁺] increase inhibits the PMN transmigra-
tion as well as the increase in permeability, but not PMN adhesion. Furthermore, PMN adhesion and transmigration in response to chemoattractants FMLP or LTB$_4$ resulted in phosphorylation of endothelial MLCK and pharmacological inhibition of MLCK decreased transmigration (68, 95, 199). The importance of MLC-mediated contraction is further supported by findings that stabilization of actin filaments with phalloidin or jasplakinolide inhibits leukocyte transmigration across the endothelium (12, 98). These studies suggest a model by which leukocyte-endothelial interactions during adhesion and transmigration events signal the activation of endothelial cells, resulting in endothelial contraction, junctional remodeling, and impaired barrier, conditions that would facilitate transmigration. Importantly, the studies implicate the involvement of Ca$^{2+}$ and MLC, both of which are elevated by oxidant stress, suggesting that these signals may be key regulatory mechanisms in transmigration.

Oxidant stress has been shown to stimulate the production and release of platelet-activating factor (PAF) in endothelial cells (138). Once released extracellularly, PAF becomes bound to the endothelial surface and mediates PMN adhesion (138). PAF is a potent phospholipid autacoid implicated in a number of pathophysiological conditions, including inflammation, ischemia-reperfusion injury, and shock (125). The administration of a PAF-receptor antagonist (WEB-2086) or a PAF antagonist (BN-52021) abolishes leukocyte recruitment mediated by perfusion of xanthine/xanthine oxidase in the intact lung (63) and pancreas microvasculature (100), supporting the idea that PAF production is a key component contributing to oxidant-mediated endothelial dysfunction. Recent reports have indicated that oxidant stress produces PAF-like phospholipids from phosphatidylcholine in greater abundance and with greater potency than PAF (152, 228). It is evident that PAF as well as PAF-like phospholipids are critical factors in the pathophysiology of vascular endothelial dysfunction under oxidant stress conditions. It will be important to understand the mechanisms by which ROS regulate the production and release of these phospholipid mediators.

CONCLUSIONS AND FUTURE PERSPECTIVES

Oxidant stress, a pervasive condition of increased amounts of ROS, constitutes a serious pathophysiological factor for a wide variety of vascular-based disorders. It is apparent that its effects on the vascular endothelium are multifactorial, and two significant consequences are endothelial barrier dysfunction and increased adhesion for leukocytes. At present, our understanding of how ROS increase endothelial permeability and leukocyte extravasation is not well defined. Furthermore, despite much available information regarding signaling pathways that are activated by ROS, the causal relations between the signals generated and the functional response have not been rigorously investigated. Several aspects of oxidant stress-mediated endothelial dysfunction should be emphasized for future investigation. 1) For example, what are the molecular mechanisms by which ROS disassemble/assemble the tight and adherent junctions? The importance of the Rho GTPases in regulation of the actin cytoskeleton is evident. Does oxidative stress regulate the Rho GTPases? Furthermore, oxidant-mediated remodeling of the actin cytoskeleton is implicated in the loss of junctional organization and stability, but this hypothesis has not been vigorously tested. Several protein kinases have been identified to regulate barrier function; yet specific roles and targets of these kinases (and phosphatases) remain unresolved. 2) An exciting area of study is defining the molecular communication or interactions between the endothelial cell and the transmigrating leukocyte under oxidant stress conditions. This is particularly important in light of the fact that mechanisms governing leukocyte extravasation are highly stimulus, and likely tissue, specific. It has been shown that the adherent or transmigrating leukocyte can induce endothelial cell activation responses, including remodeling of endothelial junctions and the actin cytoskeleton. The functional significance and the regulatory mechanisms engaged by these interactions during oxidant stress have yet to be determined. 3) As shown by Kokura et al. (124), a redox imbalance in endothelial cells results in both a transcription-independent and -dependent surface expression of different endothelial cell adhesion molecules, suggesting that oxidant stress induces acute and chronic phases of leukocyte adhesion to the endothelium. The control of these different facets of leukocyte adhesion likely engages different regulatory mechanisms. Future studies are needed to identify and define the molecular events by which the endothelium regulates leukocyte adhesion and barrier function in the context of acute (minutes) vs. chronic (more than several hours) oxidant stress. This information is important in fully understanding the mechanisms of oxidant stress-induced endothelial dysfunction.

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