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# ROS-activated calcium signaling mechanisms regulating endothelial barrier function

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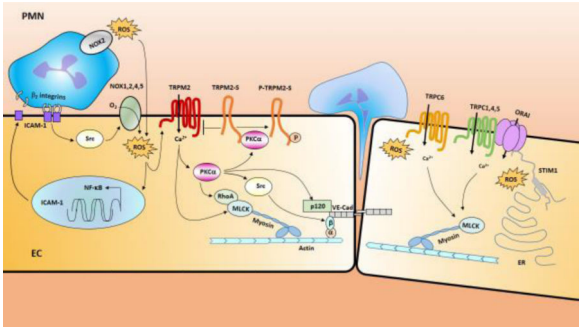
## Abstract

Increased vascular permeability is a common pathogenic feature in many inflammatory diseases. For example in acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS), lung microvessel endothelia lose their junctional integrity resulting in leakiness of the endothelial barrier and accumulation of protein rich edema. Increased reactive oxygen species (ROS) generated by neutrophils (PMNs) and other inflammatory cells play an important role in increasing endothelial permeability. In essence, multiple inflammatory syndromes are caused by dysfunction and compromise of the barrier properties of the endothelium as a consequence of unregulated acute inflammatory response. This review focuses on the role of ROS signaling in controlling endothelial permeability with particular focus on ALI. We summarize below recent progress in defining signaling events leading to increased endothelial permeability and ALI.

## Graphical Abstract




## Introduction

Inflammatory syndromes, as an example - acute lung injury (ALI) and its agonal variant acute respiratory distress syndrome (ARDS) are the result of severe disturbances on the endothelial barrier[1, 2]. The worsening loss of endothelial barrier function is thought to be

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the result of an unregulated acute inflammatory response following an initiating event, such as sepsis. If this unchecked it leads to activation of the acute inflammatory response at a systemic level affecting the permeability of multiple vascular barriers, including lungs. One of the earliest manifestations is activation of pulmonary endothelial cells (EC) and macrophages (MΦ), upregulation of adhesion molecules, and production of cytokines and chemokines that induce a massive sequestration of PMNs within the pulmonary microvasculature, resulting from inappropriate adhesion of PMNs and other leukocytes with the hyper-adherent endothelium. These inflammatory cells transmigrate across the endothelium into tissue and release a variety of cytotoxic and proinflammatory compounds, including reactive oxygen species (ROS), proteolytic enzymes, and nitrogen species, cationic proteins, lipid mediators, and additional inflammatory cytokines[3]. Other cells remain sequestered in the microvessel where they produce the same toxic substances that also induce endothelial injury. In this short review, we will focus on the role of ROS-mediated signaling in disrupting the barrier properties of the endothelium in the context of ALI (used here as a paradigm for other inflammatory disease associated with inflammation). We will focus on recent progress in studies on the signaling event underline the interaction of PMN and EC and its consequences on endothelial barrier function.

## Constitution of endothelial barrier and regulation of EC permeability

The endothelium functions as a semipermeable barrier regulating tissue fluid homeostasis and transmigration of leukocytes and providing essential nutrients across the vessel wall (see reviews [4, 5]). Transport of plasma proteins and solutes or transmigration of leukocytes across the endothelium involves two different routes: one transcellular, via caveolae-mediated vesicular transport, and the other paracellular, through interendothelial junctions[5]. Briefly, the transcellular or transcytosis pathway is responsible for the transport of albumin across the endothelial barrier via fission of plasma membrane macrodomains enriched with caveolin-1 (Cav-1), caveolae, from the luminal surface of the endothelial cell followed by transport of caveolar vesicles to the basal surface. This finely regulated process is essential for transport of albumin, albumin-bound ligands, and hormones and for control of tissue oncotic pressure in the normal continuous endothelium and the interstitial space[5]. The paracellular permeability of the endothelial barrier is maintained by the interendothelial junctions, the structures that by connecting adjacent endothelial cells into the monolayer restrict the transport of plasma proteins of the size of albumin from the vessel lumen to stroma[5]. Two general types of interendothelial junctions present in the endothelium, tight junctions (TJs) and adherence junctions (AJs), contribute to maintenance of the endothelial barrier. The molecule primarily responsible for AJs is the transmembrane hemophilic adhesion molecule, vascular endothelial (VE)-cadherin[6]. Homotypic formation of firm EC–EC junctions is maintained by VE cadherin with its cytoplasmic domain binding to β-catenin, and α-catenin which is also linked to the actin cytoskeleton[6]. The linkage between VE-cadherin-based adheren junctional complex and the actin cytoskeleton contributes to the strong adhesion[7]. While the transcellular permeability is mainly regulated by caveolae-mediated transcytosis, there are two independent mechanisms involved in regulating paracellular endothelial permeability: destabilization of AJs via phosphorylation of constituents of AJs, which in most cases leads to VE-cadherin internalization, and activation

of acto-myosin contractility accompanied by reorganization of the actin cytoskeleton into stress fibers, thus applying mechanical forces to AJs that break apart the junctions (see more details in[5]). Below we describe the paracellular permeability of lung EC regulated by ROS-mediated signaling as there is far more known about this pathway than the transcytosis/transcellular which remain enigmatic despite its potential importance in regulating tissue fluid balance and pathophysiological relevance in inflammatory diseases.

## Participation of ROS in regulating lung EC barrier function

At physiological concentrations, reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes including regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production, and signal transduction from membrane receptors in various physiological processes [8]. Many of the ROS-mediated responses protect the cells against oxidative stress and reestablish “redox homeostasis” [8]. Increased reactive oxygen species (ROS) generated in tissue during inflammation play an important role in the development of not only the full-blown inflammatory disease but also its chronicity; this case has been strongly made for ALI and its progression to ARDS[9-13]. Biologically important ROS include superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^-$), and hypohalous acids such as HOCl[3]. There are many potential sources of ROS, including phagocytes (PMN and MΦ) and non-professional phagocytes including endothelium, epithelium, fibroblasts, and smooth muscle cells that express NADPH oxidase (NOX) are capable of generating physiologically important amounts of ROS[3]. Other sources of ROS include mitochondrial electron transport chain, cytochrome P450, and xanthine oxidase[3]. Leukocytes, principally PMN and MΦs, are generally considered to be the most prodigious source of ROS in the context of ALI/ARDS[3] (**Figure 1**). PMN and MΦ express NOX2 that can generate ROS in substantial amounts[14]. The large numbers of activated PMN in the lung in ALI has focused attention on these phagocytes as a major source of ROS and disease progression. While major mechanisms of scavenging of ROS exist and are important, these are overwhelmed in the face of intense barrage of ROS generated during the inflammatory crisis. To add to the “toxic soup”, diverse pro-inflammatory mediators lipopolysaccharide (LPS), cytokines, chemokines, complement fragments, clotting fragments, and lipid mediators are released setting up multiple amplification loops and further activating PMNs and other inflammatory cells to generate ROS[3]. The ECs are also involved in this amplification process. There are four isoforms, NOX1, NOX2, NOX4 and NOX5, expressed in endothelial cells[14-16]. A critical *in vivo* role of endothelial NOX2[17] in oxidant-mediated endothelial barrier dysfunction has been described using transgenic mice overexpressing NOX2 in the endothelium[15, 18-22]. In response to inflammatory mediators, endothelial overexpression of NOX2 led to increased ROS production compared to wild type, whereas knockdown of NOX2 with siRNA reduced this effect [23, 24].

In addition, post translational mechanisms are important in the generation of ROS and likely participate in the loss of endothelial barrier function. A key step in NOX2-derived ROS production is the phosphorylation on serines by PKC isoforms such as PKCδ[25]. NOX4 and NOX5 isoforms, abundant in ECs[15], their roles has not been well investigated. Interesting recent evidence shows that NOX5, also present in ECs, can be activated by $Ca^{2+}$

[15], raising the intriguing possibility that $Ca^{2+}$ entry may be important in activation of ROS signaling through this isoform; thus, $Ca^{2+}$-mediated signaling pathway and ROS-mediated signaling pathway may interact to synergistically increase EC permeability.

## ROS-mediated signaling in controlling EC paracellular permeability

ROS mediated EC barrier dysfunction by directly attacking on various cellular components including membrane, cytosolic, and nuclear lipids and proteins through oxidative modifications [3]. Below we discuss the signals activated by ROS that underlie the increased permeability response.

### 1. Role of ROS in initiating $Ca^{2+}$ signaling in ECs

ROS generation by activated MΦ, PMNs, ECs, and other inflammatory cells sequestered in lungs is a critical factor mediating increased lung EC permeability in the setting of sepsis [9-13]. An increase in cytosolic $Ca^{2+}$ precedes changes in endothelial cell shape and the opening of AJs [26-29]. ROS production from endothelium also increases cytosolic $Ca^{2+}$ mediating increased lung EC permeability[30, 31]. However, precisely how oxidants influence $Ca^{2+}$ signaling remains unclear. With the discovery of oxidant-sensitive transient receptor potential (TRP) channels including TRPC3,4,5,6, TRPM2, TRPMV1, TRPA1which are also permeable to $Ca^{2+}$ [32-41], we now have better understanding of the mechanisms underlying the link between oxidant stress and EC permeability. TRP family has emerged as a predominant regulator of non-selective cation channels that mediate $Ca^{2+}$ entry in endothelial cells[26, 42-44]. Of these TRP channels, TRPC, TRPM and TRPV are more studied in endothelial cells[30, 42, 45-47]. Like other TRP channels, these channels also contains six transmembrane domains with a pore forming unit located between transmembrane domain 5 and 6[44]. TRPC and TRPM family members also contain proline -rich sequences in the C-terminal region of TRP domain designated as TRP box 2 which binds phosphatidyl insositol phosphates, such as PI (4, 5) $P_2$ [4, 44, 48, 49]. TRPC and TRPV family members also contain 3-4 ankyrin repeats at their N-terminus[44, 50]. The functional TRP channel is a tetramer, and may be composed of homo- or hetero- tetramers formed within the same sub-family[44]. Recent studies in our laboratory along with other studies strongly indicate that TRPC (including TRPC1, TPPC3, TRPC4 and TRPC6) act as store operated $Ca^{2+}$ (SOC) entry or receptor-operated calcium (ROC) entry channels that participate in vascular permeability regulation[51-55]. The important association between calcium release from the endoplasmic reticulum (ER) and calcium entry across the plasma membrane had been recognized for about 30 years[56]. But the nature of coupling between ER and plasma membrane that underlies SOC entry has been poorly understood until the recent identification of ORAI (calcium release-activated calcium channel protein 1), the pore of $Ca^{2+}$ release-activated $Ca^{2+}$ channel (CRAC) and stromal interaction molecule 1 (STIM1), a ROS sensor[57], which also senses the depletion of $Ca^{2+}$ from the ER, and then oligomerizes, translocates to junctions adjacent to plasma membrane, helps to organize ORAI or TRPC channels in the membrane[58]. The following section we will focus on the roles of the most-studied TRPC6, TRPM2 and STIMI on ROS-mediated $Ca^{2+}$ signaling in ECs.

TRPC6 is highly expressed in human and mouse lung endothelial cells[27, 29, 59-61] and can be activated by ROS ($H_2O_2$)[62, 63]. In TRPC6-expressing HEK293T cells, $H_2O_2$ significantly stimulated $Ca^{2+}$ entry in a dose-dependent manner[62]. Electrophysiological experiments showed that $H_2O_2$ significantly increased TRPC6 channel open probability and whole-cell currents[62]. Additionally, $H_2O_2$ stimulated a dose-dependent constriction of the aortas from wild type but not from the vessels of TRPC6 deficient mice[63]. These results suggest a new signaling pathway mediated by ROS-TRPC6 in controlling vessel contraction [63]. TRPC6 is also activated by DAG independently of store depletion and is therefore referred to as receptor-operated channels (ROC) [27, 29, 52, 64]. Several studies showed that TRPC6 plays an important role in regulating endothelial permeability[29, 59, 60]. Initially, flufenamic acid, a TRPC6 activator, was shown to increase water conductivity in frog mesenteric vessels[59, 65]. Subsequent studies showed that siRNA-induced suppression of TRPC6 channel in human pulmonary artery endothelial cells decreased endothelial cell permeability in response to thrombin[27]. Recently we showed that TRPC6 plays a key role in signaling both LPS-induced lung vascular permeability and inflammation[29]. Tauseef et al showed that LPS, which is well-known to induce ROS, resulted in DAG production which activated TRPC6 [29]. Activated TRPC6 induced MLCK (myosin light chain kinase) activity that by stimulating actomyosin cross-bridging mediates endothelial cell contraction leading to increased lung vascular permeability[29]. Additionally, activated MLCK promoted the interaction of myeloid differentiation primary response gene (MyD88) with inteterleukin receptor −1 associated kinase 4 which is involved in triggering NF-kB signaling and pulmonary inflammation downstream of TLR4 [29]. Thus, TRPC6 appears to be at the center of the signaling pathways mediating ALI owing to its dual role in increasing lung vascular permeability and mediating TLR4 signaling[29]. In a recent study we also showed that TRPC6 was required for transendothelial migration i.e. diapedesis downstream of PECAM (Platelet endothelial cell adhesion molecule-1) homophilic interactions[66]. We showed that TRPC6 colocalized with PECAM and regulated PMN transendothelial migration (TEM). Expression of dominant-negative TRPC6 or shRNA knockdown in endothelial cells arrested PMN apically over the junction while selective activation of endothelial TRPC6 with hyperforin 9 induced TEM even in the absence of PECAM[66]. Consistently, in a croton oil-mediated acute ear inflammation model, mice lacking TRPC6 exhibited a profound defect in neutrophil TEM with no effect on leukocyte trafficking[66] (**Figure 2**). It is interesting that ROS activates TRPC6 channels via modification of thiol groups of intracellular proteins and this cysteine oxidation-dependent pathway not only stimulates the TRPC6 channel by itself but also sensitizes the channels to DAG [62]. Thus, ROS may enhance LPS-TRPC6 pathway in a DAG dependent manner in regulating EC permeability.

STIM1, a type 1A single transmembrane protein originally identified as a tumor suppressor protein, has been established as a $Ca^{2+}$ sensor within the ER stores[57, 67]. STIM1 contains N-terminal EF hand domain, a $Ca^{2+}$ binding domain, a sterile α motif or SAM domain, a single transmembrane domain (TM), an ezrin-radixin-moesin (ERM) domain, a serine-proline rich region (S/P-region) and a lysine rich region (E-region)[57, 58, 68]. STIM1 dimerizes before clustering and the activation spices is the dimer. When bound with $Ca^{2+}$ in ER lumen, STIM1 exists as an individual unit or a monomer. Upon store depletion the EF

hand domains and SAM domains from different STIM1 protein aggregate and form multimeric puncta which leads to its interaction with store operated $Ca^{2+}$ channel and activation of SOC entry[28, 58, 67]. STIM1 has been shown to regulate TRPC channels such as TRPC1, 4,5, and ORAI channels which are the important constituents of SOC and CRAC respectively[69, 70]. STIM1 has been shown to act as ROS sensor[57]. ROS were shown to induce STIM1 aggregation, STIM translocation to ER–plasma membrane junctions and activation of ORAI channels without $Ca^{2+}$ store depletion[57, 71]. ROS-induced S-glutathionylation of Cys56 in the amino terminus of STIM1 decreases $Ca^{2+}$ binding by the EF-hand domain and triggers STIM1 activation[57, 71]. Oxidant stress led to a phenotypic shift in $Ca^{2+}$ mobilization from an oscillatory to a sustained elevated pattern via CRAC-mediated capacitive $Ca^{2+}$ entry, and STIM1- and ORAI-deficient cells are resistant to oxidant stress[71]. These experiments reveal that Cys56 is a sensor for oxidant-dependent activation of STIM1 and demonstrate a molecular link between oxidant stress and $Ca^{2+}$ signaling via the CRAC channel[71]. A subsequent study report an alternative role of Cys56 in the mechanism of STIMI activation [72]. It was discovered that STIM1 oligomerization and SOC were modulated by the endoplasmic reticulum (ER) oxidoreductase ERp57 which interacts with the ER luminal domain of STIM1[72]. The interaction involving two conserved cysteine residues, C49 and C56. SOC is inhibited in C56 mutants of STIM1[72]. Thus, the role of Cys56 in activationg STIM1 is not completely clear. Our laboratory showed that Cav-1 forms an important link between TRPC1 and inositol 1,4,5-trisphosphate receptor 3 ($IP_3R3$) [53]. We found that Cav-1 scaffold domain (CSD) interacts with both TRPC1 and $IP_3R3$, and thereby regulates SOC-mediated entry [53]. The rise in intracellular $Ca^{2+}$ through these TRPC channels may activate key signaling pathways, resulting in MLC-dependent endothelial cell contraction and disassembly of vascular endothelial cadherin (VE-cadherin) at the AJs and lead to increased vascular permeability [64, 73]. Thus, oxidant-sensitive STIMI1 may form a complex with TRPCs and/or ORAI to regulate ROS-mediated $Ca^{2+}$ signaling in regulating EC permeability (**Figure 2**).

Recently we have described the important role of the redox-sensitive $Ca^{2+}$ permeable cation channel TRPM2 (transient receptor potential melastatin 2) in regulating EC permeability following oxidative stress[30, 46]. The method of TRPM2 activation and its role is described in **Figure 2**. TRPM2 is an oxidant-sensitive cation channel expressed in endothelial and phagocytic cells[30, 35, 47, 74-78]. Channel opening after exposure to oxidants is induced by binding of the intracellular second messenger adenosine diphosphoribose (ADP-ribose) or related molecules to the Nudix box sequence[32, 78-80] in the carboxyl-terminal sequence of TRPM2[77]. The mechanism of TRPM2 channel activation involves $H_2O_2$, produced during oxidative stress, which activates the nuclear and mitochondrial production of ADP-ribose [32, 35, 80] that binds to the TRPM2 Nudix box sequence, and signals TRPM2-mediated $Ca^{2+}$ entry[32, 35, 78-81]. We observed that suppressing endogenous TRPM2 expression or activity by small interfering RNAs, a specific anti-TRPM2 blocking antibody, overexpression of TRPM2-S isoform, and poly-ADP-ribose polymerase inhibitors prevented the generation of ADP-ribose[30, 35, 82, 83] and abolished the $H_2O_2$-induced $Ca^{2+}$ influx via TRPM2 channel and, importantly, prevented the increase in endothelial permeability[30].

In addition to the full-length $Ca^{2+}$ permeable channel protein TRPM2 (i.e., TRPM2-L), several TRPM2 isoforms have been identified, potentially the most important of these is the short splice variant (TRPM2-S)[84]. TRPM2-S lacks the carboxyl terminus of the longer forms including the putative $Ca^{2+}$-permeable pore, and thus does not function directly as a $Ca^{2+}$ channel but interacts with TRPM2 in the plasma membrane[30, 46], negatively regulates $Ca^{2+}$-channel activity of TRPM2[46, 82, 84]. We observed that overexpression of TRPM2-S isoform inhibited the $H_2O_2$-induced $Ca^{2+}$ influx via TRPM2 channel and prevented the increase in endothelial permeability[30]. Thus, TRPM2/TRPM2-S interaction is pathophsyiologically important and play a key role in the mechanism of increased lung vascular permeability and PMN tissue infiltration under oxidant stress.

## 2. ROS-$Ca^{2+}$-PKCα pathway in signaling EC permeability

An earlier review well summarized potential mechanisms underlying ROS-mediated increased endothelial cell permeability that involved a serine and threonine-specific protein kinase family protein kinase C (PKC) such as PKCζ[4]. Among PKC family, PKCα is a member activated by $Ca^{2+}$ and the second messenger DAG[85]. $Ca^{2+}$ binding to the "$Ca^{2+}$-binding loops" of PKCα is required for its translocation from the cytoplasm to the plasma membrane and for PKCα activation [86, 87]. We showed that TRPC6-induced $Ca^{2+}$-entry was required for inducing PKCα activity [27]. Intriguingly, we found that PKCα has an important role in signaling the observed oxidant-induced TRPM2 activation[30, 46] raising the possibility that TRPC6 may induce TRPM2. We identified a high-affinity binding site for PKCα in the N-terminal domain of TRPM2, which regulated the interaction of PKCα with a TRPM2 isoform. $H_2O_2$ induced rapid co-localization of PKCα with TRPM2-S (the short-splice variant of TRPM2) that was not observed when TRPM2 was knocked down[25,37]; it is therefore likely that PKCα regulates TRPM2-induced $Ca^{2+}$ entry and endothelial permeability through PKCα phosphorylation of TRPM2-S (**Figure 2**).

PKCα activation is known to increase microvascular permeability as first described by us in 1990 [88]. PKCα was found to change its intracellular distribution on $H_2O_2$ exposure [89] and signal $H_2O_2$-induced increase in endothelial permeability[12, 90] through mediating the phosphorylation of p120-catenin [91] (**Figure 2**). On the other hand, PKCα mediate activation of cSrc [92, 93] which directly or indirectly leads to tyrosine phosphorylation of VE-cadherin and β-catenin, resulting in adherens junction destabilization[94, 95]. PKCα also modulates RhoA GTPase activation by phosphorylation of the upstream regulators of RhoA, the Rho guanosine diphosphate (GDP) dissociation inhibitor GDI-1 and p115RhoGEF [5, 96]. PKCα phosphorylates GDI-1 at Ser96, thus reducing GDI-1 affinity for RhoA that favors the exchange of GDP to GTP by p115RhoGEF[5]. RhoA facilitates phosphorylation-induced inhibition of myosin light chain phosphatase (MLCP) by activating Rho kinase (ROCK)[97, 98]. The inhibition of MLCP accompanied by the $Ca^{2+}$/calmodulin-dependent activation of MLCK leads to phosphorylation of MLC and induces acto-myosin contraction in response to proinflammatory mediators such as thrombin and histamine [5, 99-101]. In this regard, PKCα cooperates with MLCK to elicit a coordinated spatial activation of RhoA and global reorganization of the actin cytoskeleton, resulting in endothelial barrier dysfunction[5] (**Figure 2**). Thus, ROS-TRPM2-PKCα may form an important pathway in ROS-mediated controlling of EC permeability (**Figure 2**).

### 3. ROS -ICAM-1-Src circuit in controlling EC permeability

It is well known that oxidative stress is also initiated by activated PMNs [3, 12] following the adhesion and sequestration of PMNs. The production of oxygen metabolites such as $H_2O_2$ increases endothelial adhesivity of PMNs and lung vascular endothelial permeability[12, 82-84], both critical factors governing formation of tissue edema and PMN extravasation. Endothelial cell surface expression of ICAM-1, the counter-receptor for PMN $\beta_2$-integrins, results in adhesion of activated PMNs to ECs and subsequently, PMN transmigration into tissue[102] (**Figure 2**). ROS stimulate the activation of NF-κB[103], which in turn controls the expression of key genes involved in mediating lung vascular inflammation and injury[103-105]. In resting cells, NF-κB proteins are sequestered in the cytoplasm through their tight association with IκB proteins. NF-κB activation relies on IκB phosphorylation and degradation, such that the freed NF-κB proteins translocate into the nucleus and regulate the expression of multiple inflammatory target genes[106]. As NOX2 is an essential regulator of oxidative stress-induced NF-κB activation[107], NOX2 activation induces upregulation of ICAM-1 expression through NOX-dependent NF-κB activation, it is possible that increased ICAM-1 expression promotes additional PMN/EC interaction, and hence amplifies TRPM2-mediated $Ca^{2+}$-entry activating a feed-forward mechanism and leading to severe lung injury (**Figure 2**). In endothelial cells, the increase in intracellular $Ca^{2+}$ was shown to induce exocytosis of Weibel-Palade bodies (WPB) [108-111] (specialized secretory vesicles containing preformed proteins such as P-selectin which are released upon cell stimulation) [112-117]. P-selectin (CD62P) is a member of the selectin family of cell adhesion molecules[118] and play a key role in PMN rolling adhesion to activated EC [119]. But the effect of ROS in regulating exocytosis of WBP remain unclear. One group reported that the expression of P-selectin on the surface of endothelial cells was accompanied by qualitatively parallel increases in ROS generation [120]. Both P-selectin expression and ROS generation were inhibited, dose dependently, by the exogenous administration of disparate cell-permeable antioxidants and also by the inhibition of either of the known membrane-associated ROS-generating enzymes NADPH oxidase or xanthine oxidase [120]. In contrast, it was found that $H_2O_2$ inhibit thrombin-induced exocytosis of granules from endothelial cells by inhibiting N-ethylmaleimide sensitive factor (NSF), a protein that regulates membrane fusion [121]. It would be important to determine whether ROS-mediated $Ca^{2+}$ signaling up-regulates membrane surface expression of P-selectin through exocytosis of WPB and therefore up-regulated interaction of PMN and EC and form another pathway in amplifying TRPM2 signaling.

Recent studies have pointed to a key signaling role of ICAM-1, beyond its function as an adhesive protein regulating leukocyte adhesion and transmigration [122-124]. ICAM-1 engagement was shown to lead to activation of tyrosine kinase Src [95]. PMN binding to ICAM-1 or direct ICAM-1 cross-linking induced Src activation[125]. c-Src is an important upstream kinase that regulates NADPH oxidase-induced ROS production [7, 126] and the cytoplasmic tyrosine kinase c-Src induces NADPH oxidase activation [127, 128]. Exposure of cultured ECs to LDL stimulated ROS formation, which was prevented by Src kinase inhibitor PP1 [129]. Src mediates phosphorylation of p47phox and its translocation to the membrane in hyperoxia-induced activation of NADPH oxidase in lung ECs [130]. Src may also activate NADPH oxidase indirectly through another kinase such as PKCζ[131, 132].

Bearing in mind that the PMN $\beta_2$-integrin interaction with ICAM-1 in ECs can activate ICAM-1 signaling [122-124], thus, NOXICAM-1-Src may form a key circuit in controlling EC permeability through ROS-mediated TRPM2 activation (**Figure 2**). On the other hand, the cytoplasmic tyrosine kinase c-Src may directly regulate EC permeability by transducing signals that mediate AJ destabilization and acto-myosin contractility [5, 94, 133-137]. c-Src activation directly or indirectly leads to tyrosine phosphorylation of VE-cadherin and β-catenin, resulting in AJ destabilization as mentioned above[94, 95]. Transmigration of leukocytes is associated with c-Src dependent phosphorylation of VE-cadherin at Tyr658 and Tyr731 and reduces VE-cadherin binding to p120-catenin and β-catenin[95]. Thus, ROS -ICAM-1-Src circuit may control lung EC permeability through both promoting the interaction of PMN and EC and initiating TRPM2 activation (**Figure 2**).

## Role of ROS signaling in controlling EC transcellular permeability

There is little information about the role of ROS in controlling the transcellular permeability of EC[4, 5]. As mentioned earlier, the increase in intracellular $Ca^{2+}$ in EC was shown to induce exocytosis of WPB including P-selectin upon cell stimulation[112-117], whether caveolar transcytosis of albumin requires a similar exocytic stimulus is unknown[4, 5]. It is important to clarify whether ROS control EC paracellular permeability via activating $Ca^{2+}$ signal through redox-sensitive channel such as TRPM2.

## Concluding Remarks

Increased ROS generated in tissue plays an important role in increasing endothelial permeability that progresses to inflammatory syndromes such as ALI/ARDS. Although in essence, these syndromes are caused by dysfunction and compromise of the barrier properties of endothelium as a consequence of an unregulated acute inflammatory response, the precise mechanisms of oxidant-mediated disruption of endothelial barrier remain elusive. With the discovery of oxidant-sensitive $Ca^{2+}$ permeable TRP channels such as TRPM2 and TRPC6, and sensors for both $Ca^{2+}$ and ROS such as STIM1, the mechanism underlying ROS mediated $Ca^{2+}$ signaling in regulating EC permeability become clearer. Our most recent observations imply the potentially crucial role of endothelial redox sensitive TRPM2 channel activation in mediating the PMN activation-induced endothelial permeability and PMN infiltration as well as the role of PKCα – TRPM2-S pathway and ICAM1- c-Src pathway in the regulation of TRPM2 activation and TRPM2-induced increase in endothelial permeability under oxidant stress. Because both isoforms of TRPM2 (TRPM2-L and TRPM2-S) are expressed in endothelial cells, the mechanisms that regulate their expression or control of their interaction could be targeted to pharmacologically suppress endothelial permeability under oxidant stress. On the other hand, redox sensitive TRPC6 and other TRPCs such as TRPC1 which may form CRAC by connecting with ORAI and ROS sensor STIM1, may server as another important regulator in controlling EC permeability upon oxidant stress resulting from challenges such as sepsis. Thus, mechanisms that regulate these factors such as their expression or control of their interaction could be targeted to pharmacologically suppress endothelial permeability during ALI/ARDS result from oxidant stress.

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## List of Abbreviations

| | |
|---|---|
| **AJs** | adherence junctions |
| **ALI** | acute lung injury |
| **ARDS** | acute respiratory distress syndrome |
| **Cav-1** | caveolin-1 |
| **CRAC** | calcium-release-activated calcium channel |
| **CSD** | Cav-1 scaffold domain |
| **DAG** | diacylglycerol |
| **EC** | endothelial cells |
| **ER** | endoplasmic reticulum |
| **ERM** | ezrin-radixin-moesin |
| **GDI** | GDP dissociation inhibitor |
| **GDP** | guanosine diphosphate |
| **HEK293T** | |
| **ICAM-1** | Intercellular Adhesion Molecule 1 |
| **$IP_3R3$** | inositol 1,4,5-trisphosphate receptor 3 |
| **LPS** | lipopolysaccharide |
| **MLC** | myosin light chain |
| **MLCK** | myosin light chain kinase |
| **MLCP** | myosin light chain phosphatase |
| **MyD88** | myeloid differentiation primary response gene 88 |
| **MΦ** | macrophages |
| **NF-kB** | nuclear factor kappa-B |
| **NSF** | N-ethylmaleimide sensitive factor |
| **NOX** | nicotinamide adenine dinucleotide phosphate (NADPH) oxidase |

| | |
|---|---|
| **ORAI** | calcium release-activated calcium channel protein |
| **PECAM** | platelet endothelial cell adhesion molecule-1 |
| **PKC** | protein kinase C |
| **PMN** | neutrophils (polymorphonuclear leukocytes) |
| **ROC** | receptor-operated calcium |
| **ROCK** | Rho kinase |
| **ROS** | reactive oxygen species |
| **SAM** | sterile alpha motif |
| **SOC** | store operated calcium |
| **S/P-region** | serine-proline rich region |
| **STIM1** | stromal interaction molecule 1 |
| **TEM** | transendothelial migration |
| **TJs** | tight junctions |
| **TLR4** | toll-like receptor 4 |
| **TM** | transmembrane domain |
| **TRP** | transient receptor potential |
| **TRPM2-L** | transient receptor potential (TRP) subfamily M2, long form |
| **TRPM2-S** | transient receptor potential (TRP) subfamily M2, short form |
| **VE** | vascular endothelial |
| **WPB** | Weibel-Palade bodies |

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## Research Highlights

1. Increased reactive oxygen species (ROS) generated in tissue during inflammation play an important role in the development of ALI and its progression to ARDS.
2. ROS initiated $Ca^{2+}$ signaling in tissue plays an important role in increasing endothelial permeability.
3. Redox sensitive TRPM2 channel and TRPC6 and other TRPCs such as TRPC1 which form CRAC by connecting with ORAI and ROS sensor STIM1, are key players in mediating ROS-induced $Ca^{2+}$ signaling in regulating endothelial permeability upon oxidant stress.

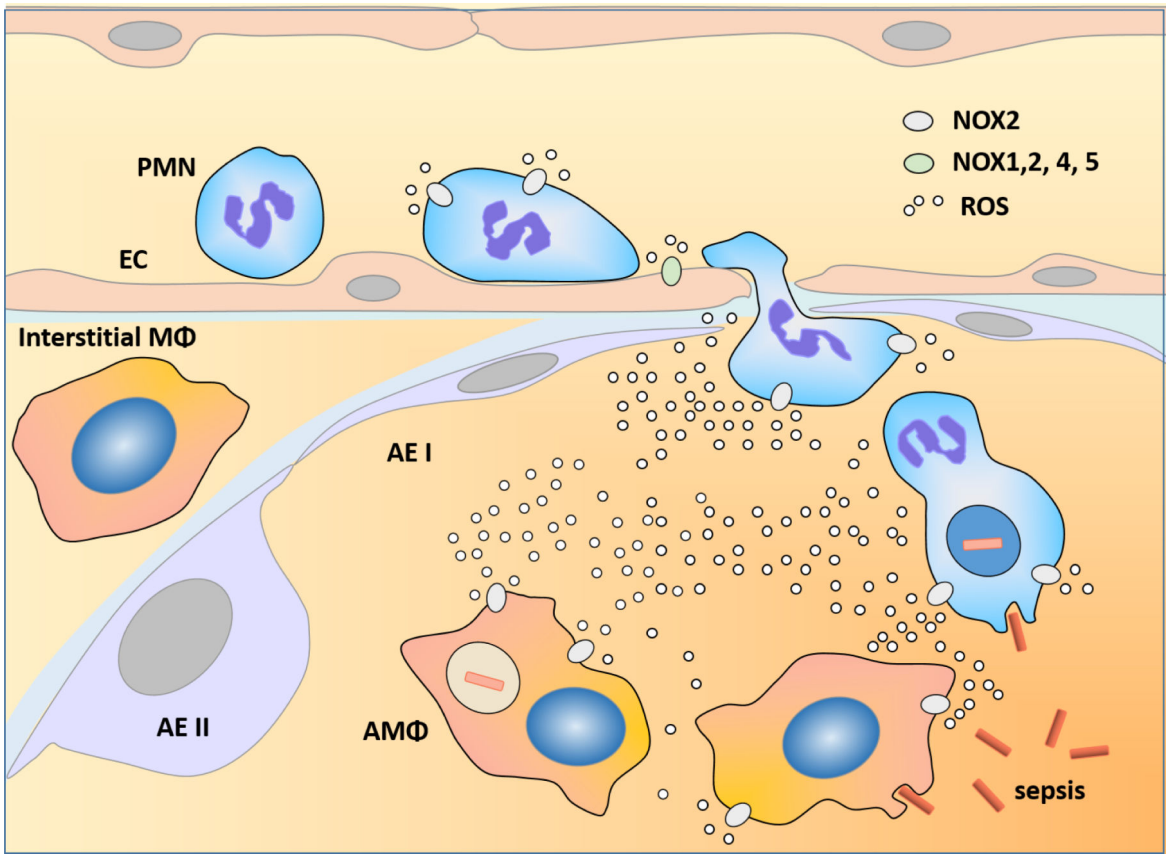


**Figure 1. NOX (NADPH oxidase) mediated production of ROS (reactive oxygen species)**

In response to an inciting event such as a result of a bacterial infection, the pulmonary macrophages (MΦ) and endothelial cells (EC) become activated and upregulate surface expression of adhesion molecules, leading to polymorphonuclear leukocytes (PMN) adhesion and subsequent transmigration from the intravascular space into the alveolus[3].. Activated alveolar MΦ (AMΦ), PMN and EC produce reactive oxygen species (ROS), however, PMN is the major source of ROS production in the context of acute lung injury and acute respiratory distress syndrome (ARDS) induced by sepsis[3]. AEI: alveolar epithelial type I; AEII: alveolar epithelial type II. NOXs are divided into two major groups here according to its distribution in cells: NOX2 group expressed in phagocytes (PMN, MΦ)[14]; NOX1,2,4,5 group expressed in ECs[14-16].

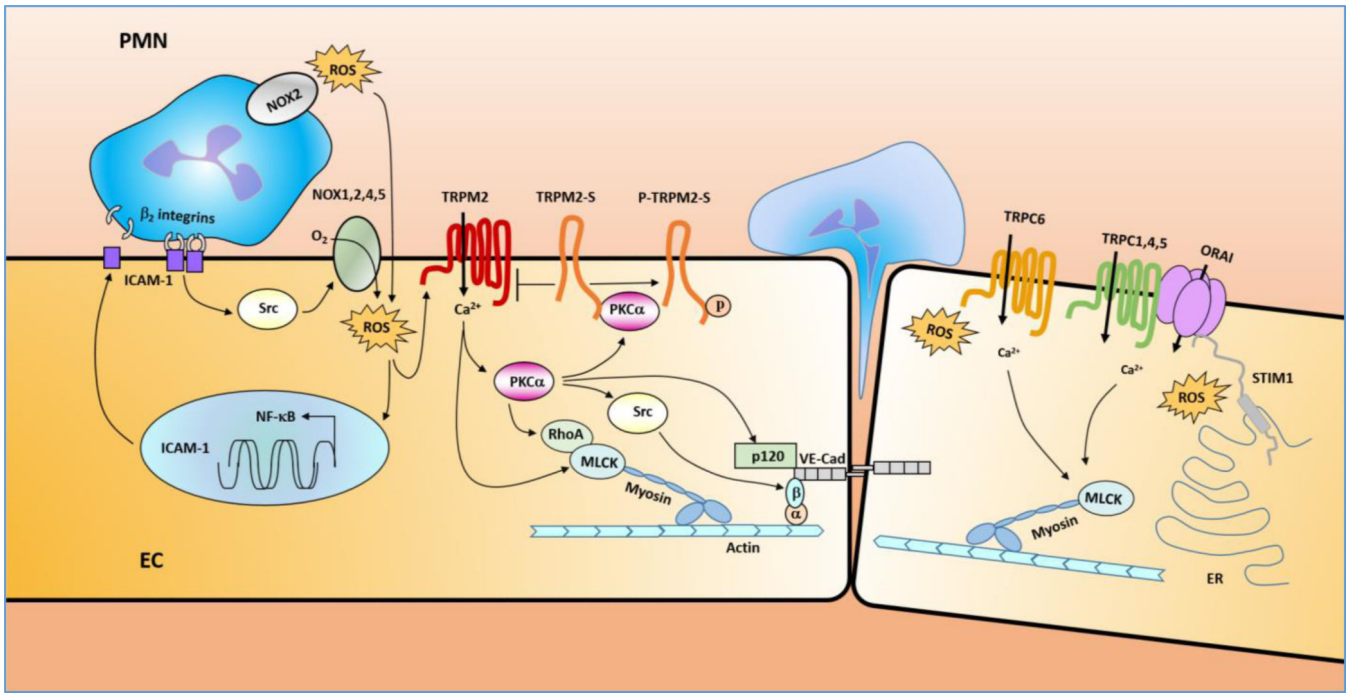


**Figure 2. Model of ROS-signaling regulating endothelium permeability**

Upon ROS challenge, endothelial cells (EC) become activated and upregulate surface expression of adhesion molecules such as ICAM1(intercellular adhesion molecule 1) through a NF-κB dependent mechanism[103-105]. The binding of the increased ICAM1 surface expression of EC with $\beta_2$ integrin of PMN induces the activation of endothelial NOXs (NOX1,2,4,5) through Src-dependent mechanism[95],[125]. Thus, activation of the NOX isoforms above and resulting endothelial ROS production stimulates NF-κB-dependent upregulation of ICAM-1 expression in ECs setting-up a positive feedback loop leading to ROS production. ROS released from both PMN NOX2 and EC NOX1,2,4,5 activated the redox sensitive $Ca^{2+}$ permeable channel TRPM2 in the endothelium and lead to $Ca^{2+}$ entry into EC and promote PKCα activation. Activated PKCα may regulate EC permeability via three major pathways: (1). Activated PKCα phosphorylates p120-catenin to induce dissociation of p120-catenin from VE-cadherin[91] leading to disruption of adherent junctions (AJs) and transmigration of PMN; (2). Activated PKCα mediates activation of cSrc [92, 93] which directly or indirectly leads to tyrosine phosphorylation of VE-cadherin and β-catenin, resulting in AJ destabilization[94, 95]; (3). Activated PKCα cooperates with MLCK to elicit a coordinated spatial activation of RhoA and global reorganization of the actin cytoskeleton, resulting in endothelial barrier dysfunction[5]. Activated PKCα also interacts with TRPM2-S and release its interaction with TRPM2 and thereby promotes TRPM2 channel activity[30, 46]. Thus, TRPM2/TRPM2-S interaction serves as a critical switch responsible for increased lung vascular permeability and PMN transmigration. On the other hand, ROS may also participate in regulating $Ca^{2+}$ influx by inducing activation of redox-sensitive TRPC6 [29, 62, 63] and TRPC1,4,5[51-55] and ORAI[58] trough redox-sensitive STIM1[57, 69, 70]. The rise in intracellular $Ca^{2+}$ through these TRPC channels or ORAI may activate key signaling pathways, resulting in MLC-dependent endothelial cell

contraction and disassembly of vascular endothelial cadherin (VE-cadherin) at the adherens junctions and lead to increased vascular permeability [64, 73].