A novel role for reactive oxygen species in the regulation of RhoA; implications for endothelial permeability and leukocyte transmigration.

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Abstract

Amir Aghajanian: A novel role for reactive oxygen species in the regulation of RhoA; implications for endothelial permeability and leukocyte transmigration. (Under the direction of Keith Burridge, PhD)

The endothelial lining of the vasculature plays a critical role in regulating the passage of fluid, macromolecules, and cells between the blood and surrounding tissues. Vascular permeability is tightly regulated and is modulated during both physiological and pathological situations. Our laboratory is interested in the mechanisms which regulate vascular permeability and the transmigration of leukocytes during inflammation. The migration of leukocytes across the endothelial barrier is called “leukocyte transendothelial migration” (TEM). Deciphering the mechanisms which regulate TEM is important to understanding and managing inflammatory diseases.

My interest in TEM is focused on pathways which involve small GTPases. My specific interest is in the role of Rho GTPases and their regulation by reactive oxygen species (ROS) during leukocyte TEM. Although ROS have been largely seen as mediators of oxidative damage, more recently, ROS have been recognized as necessary components of cell signaling pathways. Importantly, ROS have been shown to play an important role in regulating vascular permeability and TEM.

In this dissertation, I show that physiological levels of ROS can directly activate RhoA in cells. *In vitro* studies had previously identified two critical cysteine residues in the nucleotide binding pocket of RhoA that are oxidatively modified by ROS. My work showed that this oxidative regulation of RhoA can occur in a cellular context. Before these studies, the regulation of small GTPases had almost exclusively focused on regulatory proteins. Importantly, my work identifies direct oxidative modification as a novel way to regulate RhoA activity.

I extended this work by investigating the regulation of RhoA by ROS in the context of leukocyte TEM. Based on these findings and my previous work, I hypothesized that leukocyte adhesion to endothelial cells initiates ROS generation and the direct activation of RhoA to promote leukocyte TEM. In preliminary studies, I found that crosslinking of a cell adhesion molecule (ICAM-1) stimulates RhoA and Rac1 activation. In addition the activation of RhoA appears to be dependent on ROS, as seen by studies of redox-insensitive mutants. The work presented in this dissertation lays the framework for future studies on the role of ROS during leukocyte adhesion and TEM.

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List of Abbreviations

EC	Endothelial cell
ESL-1	E-selectin ligand 1
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
IRI	Ischemia-reperfusion injury
LFA-1	Leukocyte function-associated antigen 1
MAC-1	Macrophage-1 antigen
MAPK	p38 Mitogen-activated protein kinase
PBD	p21-binding domain
PSGL-1	P-selectin glycoprotein ligand 1
RBD	Rho-binding domain
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen-4
TEM	Transendothelial migration

Chapter 1: Introduction

Endothelial cell-cell junctions

The vascular system is a network of vessels that serve as conduits for the transport of nutrients, macromolecules and blood cells throughout the body. The luminal layer of the blood vessel wall consists of a single sheet of endothelial cells (ECs) which provide the primary physical barrier between the blood and underlying tissues. Cell junctions linking adjacent ECs are important regulators of the permeability characteristics of the endothelium and are modulated to allow selective and specific passage of blood cells and macromolecules. Opening and resealing of the junctional barrier must occur during normal physiological processes such as immune surveillance, antigen recognition, and acute inflammatory responses.

Conversely, misregulation of cell junctions can lead to pathological situations, including many chronic inflammatory diseases and edema. As such, the endothelium and its junctions play a critical role in regulating vascular function during both physiological and pathological processes. Vascular permeability is the sum of many mechanisms which either increase or decrease barrier function [1, 2]. In this introduction, I will focus on those pathways which mediate reversible, transient increases in permeability due to junctional disruption.

Vascular barrier function is not only differentially modulated in response to physiological stimuli within a given vessel, but additionally, different vascular beds have unique permeability and barrier characteristics due to morphologicallyheterogeneous endothelial junctions [3]. At two extremes of the continuum are the relatively impermeable endothelium forming the blood brain barrier, and the high endothelial venules which are sites of constitutive leukocyte transendothelial migration (TEM).

Regulation of EC junctions, and by extension, vascular permeability is provided by a variety of signaling cues. These signals function to promote normal homeostasis but are also involved in pathological changes in permeability. In this introduction I will discuss regulation of EC junctions, first in the context of permeability to fluid and small macromolecules; and second how these junctions are modified to allow leukocyte TEM during inflammation. Common pathways responsible for regulation of junctional permeability exist despite differences in the initiating signaling event. These pathways converge to increase junctional permeability by a variety of mechanisms.

Tight Junctions

Compared to the well-developed and spatially distinct tight junctions (TJs) of epithelial cells, in ECs TJs are less structured and more intermixed with the adherens junctions. Membrane-spanning junctional proteins contain extracellular domains for adhesion via homophilic interactions with adjacent cells, forming a physical barrier. Occludin was the first transmembrane protein to be identified at the TJs of both epithelia and endothelia [4]. Numerous lines of evidence point to

a central role for occludin in regulating paracellular permeability of blood vessels [5]. The claudin family of transmembrane proteins are also a major component of TJs. The over 20 proteins which make up the claudin family have a wide distribution, although claudin-5 appears to be specific to ECs [6]. The junctional adhesion molecule (JAM) proteins uniquely contribute to endothelial permeability because they can participate directly in barrier function, but can also act as receptors that interact with leukocyte integrins to regulate and direct leukocyte transmigration. Furthermore, the heterogeneity of EC junctions is reflected by differential expression of specific JAM isoforms (reviewed in Weber et al., 2007) [7]. In addition to these transmembrane proteins, there are also proteins that form a complex on the cytoplasmic face of the TJ, including ZO-1 and ZO-2. These scaffold proteins link the cytoplasmic domains of the transmembrane proteins to the actin cytoskeleton [8], as illustrated in Figure 1.

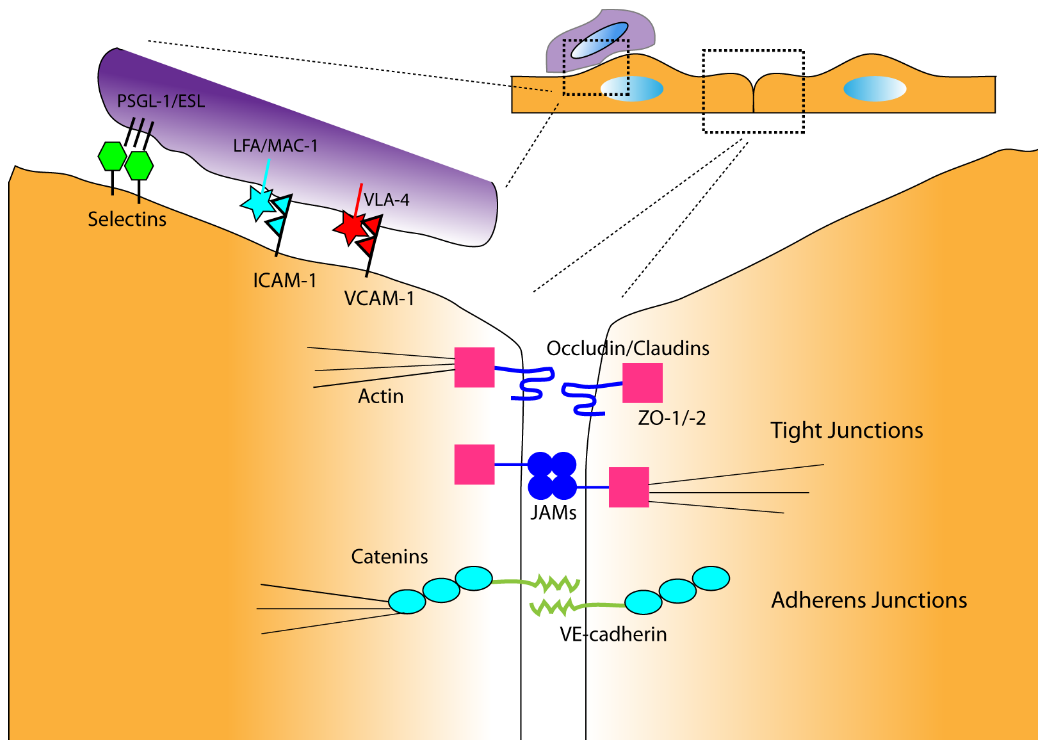


Figure 1. Intercellular interactions between endothelial cells and leukocytes. Tight junctions and adherens junctions are intermixed along the lateral surface of ECs. Extracellular domains of occludin, claudins, and JAM proteins form homophilic interactions to create tight junctions. The intracellular domains of these proteins associate with the actin cytoskeleton through ZO proteins (ZO-1/ZO-2) and other components (not shown). The major transmembrane component of adherens junctions is VE-cadherin. The cytoplasmic domain associates with the cytoskeleton via α and β-catenin and likely other unidentified components (X). p120 catenin serves a supporting role to maintain stability of the cadherin complex. Adhesive interactions between ECs and leukocytes occurs via glycoprotein ligands on the surface of leukocytes and selectins expressed by ECs. In addition, integrins expressed by leukocytes interact with cell adhesion molecules expressed by ECs (ICAM and VCAM).

Adherens Junctions

ECs also contain adherens junctions (AJs), which mediate strong adhesion between adjacent cells predominantly via the transmembrane protein VE-cadherin. The importance of VE-cadherin for barrier maintenance in ECs is underscored by the observation that injection of VE-cadherin function-blocking antibodies induces neutrophil infiltration in an *in vivo* peritonitis model [9], and vascular hyper-permeability in lung interstitium [10]. The intracellular domain of VE-cadherin binds to cytoplasmic AJ-associated proteins, particularly the catenins: α-, β-, and p120 catenin. Since their discovery, α- and β- catenin have been considered as critical linkages between cadherin cytoplasmic domains and the actin cytoskeleton. However, the dogma that this is a stable, quaternary interaction has been challenged by experiments showing that α-catenin cannot interact simultaneously with the cadherin/β-catenin complex and F-actin [11], indicating that linkage to actin may require other components [12]. Finally, p120 catenin is important during barrier maintenance, not by acting as a link to the actin cytoskeleton, but by regulating the stability of the cadherin complex, maintaining VE-cadherin's surface expression [13].

Actin Cytoskeleton

Many studies have shown that an interaction with the actin cytoskeleton is required for EC junctional integrity to anchor and reinforce junctions in ECs [14, 15]. Actin-based structures in the cell periphery (cortical actin bundles) are thought to promote junctional stability and barrier function [15]. Conversely, under conditions of increased permeability and junctional instability, cortical actin

bundles become less prominent and induction of cytoplasmic stress fibers occurs. Tension imparted by these stress fibers on junctions is thought to provide a mechanism for increasing permeability [16]. The formation of these actin structures is largely regulated by Rho family GTPases, as discussed below.

Mechanisms regulating vascular permeability

Vasoactive compounds such as thrombin, histamine and vascular endothelial growth factor (VEGF) can regulate endothelial junctions by multiple mechanisms. They can alter the architecture of the endothelial cleft by affecting junctional protein expression, localization and stability. Sometimes this occurs via protein phosphorylation. They may also activate downstream signaling pathways such as the production of reactive oxygen species (ROS) and alter the activity of Rho and Rac small GTPases, leading to changes impacting the cytoskeleton.

Expression and Stability of Junctional Proteins

There are several examples where loss of a junctional protein has been demonstrated to negatively impact barrier function. For example, loss of claudin-5 leaves morphologically normal blood vessels, but they become selectively permeable to small molecules [17]. In certain physiological instances, exposure to vasoactive compounds also causes loss of a junctional protein. For instance, histamine markedly reduces ZO-1 expression in cultured retinal ECs [18]. VEGF increases brain microvascular EC permeability by affecting occludin and ZO-1 localization at TJs, and decreasing levels of occludin expression [19]. In some situations, junctional components may be removed by proteolysis. For example,

the cytokine TGF-β upgregulates expression of matrix metalloprotease-9 (MMP-9), which results in subsequent degradation of occludin in retinal ECs [20].

One final illustration of the need for appropriate expression of junction components is provided by the interplay between p120 catenin and VE-cadherin stability and surface expression. Xiao and colleagues demonstrated that p120 catenin is required for stability and regulation of cellular VE-cadherin content [13]. Reducing levels of p120 catenin caused a dramatic dose-dependent reduction of VE-cadherin in the cell. In contrast, overexpression of p120 catenin increased surface expression of VE-cadherin by inhibiting endocytosis and endosomal degradation of cell surface VE-cadherin. Subsequent work confirmed that the interaction of p120 with VE-cadherin is required for the maintenance of endothelial barrier function [21]. This and earlier observations that vasoactive compounds such as thrombin can alter p120 catenin phosphorylation [22], suggest a mechanism linking p120 phosphorylation status, association with VE-cadherin, and destabilization of endothelial barrier function.

Protein phosphorylation and dephosphorylation

Phosphorylation is a common post-translational modification for regulating intracellular signaling. The importance of tightly regulating junctional protein phosphorylation is underscored by the observation that certain phosphatases constitutively associate with cell-cell junctions in quiescent ECs, presumably to maintain the low level of phosphorylation required for junctional integrity [22].

Occludin phosphorylation is a major mechanism for regulating TJ permeability. VEGF induces rapid phosphorylation of occludin and ZO-1 under

conditions where permeability is also increased [23]. However, there are also observations that show *in vitro* VEGF treatment leaves junctions morphologically intact, suggesting that VEGF enhances transcellular permeability [24]. Therefore, regulation of EC permeability by VEGF is complex and possibly involves two or more distinct modes of permeability. Other vasoactive compounds such as histamine and lysophosphatidic acid (LPA) also induce phosphorylation of occludin in EC lines [25]. While these studies linked phosphorylation of occludin with increased vascular permeability, a recent study proposed that occludin dephosphorylation may also decrease permeability [26]. This might indicate that phosphorylation at particular residues either promotes or inhibits permeability, and that a basal level of phosphorylation on specific residues may be required for occludin's barrier enhancing role.

Numerous studies have demonstrated the role of tyrosine phosphorylation of AJ proteins in AJ disassembly [27, 28]. The catenins are frequent targets of phosphorylation, which promotes their disassociation from VE-cadherin. ECs lacking the adhesion protein PECAM-1 have elevated levels of tyrosine-phosphorylated β-catenin, constitutive association of this pool with activated GSK-3β, and a resulting increase in proteasomal degradation of β-catenin [29]. A model was suggested in which tyrosine phosphorylation of PECAM-1 enables it to act as a scaffold, promoting recruitment of both the phosphatase SHP-2 and phosphorylated β-catenin. This facilitates dephosphorylation of β-catenin, allowing it to bind to VE-cadherin and thus promoting AJ complex reassembly [29].

VE-cadherin itself is also a target for phosphorylation downstream of vasoactive agents. Phosphorylation on two tyrosine residues present on the cytoplasmic C-terminal region of VE-cadherin, Y658 and Y731, was found to be critical to the binding of p120- and β-catenin respectively to this region [30]. Furthermore, expression of phospho-mimetic VE-cadherin mutants in an artificial cell system (CHO cells) resulted in increased permeability to HRP-IgG. However, another residue (Y685) has been recently discovered to be phosphorylated by Src [31]. In this second study, treatment with VEGF was the stimulus, so it is possible that different tyrosines are phosphorylated in different conditions. Adding another layer of complexity, VE-cadherin can also be phosphorylated on serine residues. Gavard and colleagues found that Ser665 is phosphorylated by p21-activated kinase (PAK) upon VEGF treatment and this results in β-arrestin-dependent endocytosis of VE-cadherin and promotion of EC permeability [32]. The process is initiated by Src-dependent phosphorylation and activation of the Rac1 GEF Vav2, followed by Rac1-mediated activation of its downstream effector PAK.

Rho GTPases

Rho GTPases, particularly RhoA, Rac1, and Cdc42 have a long history of being implicated in cytoskeleton regulation [33]. In the context of ECs, Rho signaling regulates cell junctions and vascular permeability by influencing actin cytoskeleton dynamics [16]. In turn, engagement of adhesion proteins influences activity of the GTPases, providing a two-way feedback mechanism [34, 35]. Although this thesis focuses on RhoA and Rac1 signaling, it should be mentioned

that Cdc42 has also been shown to have a role in regulating vascular permeability by promoting the reassembly of AJs [36].

The development of isometric tension in ECs has been suggested as a primary mechanism of regulating endothelial barrier function [37]. Very often, agents that increase vascular permeability also activate RhoA. In turn, active RhoA stimulates Rho kinase which leads to actomyosin contractility by promoting the phosphorylation of myosin light chain (MLC) directly or indirectly through inhibition of MLC phosphatase [33]. This contractility drives the formation of stress fibers and assembly of focal adhesions [38]. Rho-mediated contractility has been suggested to be a primary mechanism leading to increased permeability downstream of thrombin binding [39]. It should be noted, however, that Rho kinase also phosphorylates occludin and claudin-5, affecting their functions [40].

The case for Rac GTPase is much more controversial. Whereas activated Rac1 enhances cell–cell adhesion in epithelial cells [41], in ECs the situation is contradictory. The earliest data showed that both dominant negative and constitutively active Rac induced permeability, consistent with the level of Rac needing to be finely tuned for optimal junctional integrity [42]. Others have also demonstrated that activation of Rac, for example in response to sphingosine-1-phosphate, promoted cortical actin rearrangement and enhanced barrier function in EC monolayers [43, 44]. However, other groups have shown the opposite, with Rac activation causing increased permeability [32, 45]. VEGF treatment produced a similar effect, where downstream activation of the Rac effector PAK

induced VE-cadherin serine phosphorylation and endocytosis, leading to junctional disruption [32]. PAK family kinase activity downstream from Rac signaling can also stimulate opening of cell junctions by direct phosphorylation of MLC and subsequent induction of actomyosin contractility [46]. That active Rac in some situations increases permeability but in others decreases permeability, suggests that the input signal may be critical and that different scaffolding proteins may direct Rac signaling pathways in seemingly opposite directions.

Reactive Oxygen Species

Historically, ROS have been studied in the context of pathogen killing by phagocytic cells of the immune system, and as potentially harmful by-products of aerobic metabolism. However, more recently ROS have been recognized as important components of cell signaling pathways [47].

Numerous studies have shown that ROS can increase endothelial permeability both *in vitro* and *in vivo*, and this permeability can be inhibited by antioxidants and free radical scavengers [48]. Interestingly, Rac1 is a major component of the vascular NADPH oxidase complex [49]. Expression of constitutively active Rac1 resulted in ROS production concomitant with disruption of VE-cadherin at cell-cell junctions, tyrosine phosphorylation of α-catenin, and ultimately increased permeability [45]. It is now known that Rac-mediated ROS production leads to activation of the tyrosine kinase Pyk2, which subsequently phosphorylates β-catenin and thus destabilizes the AJ [50]. ROS generation also occurs downstream from a variety of vasoactive factors, notably VEGF. Recently, it was confirmed that VEGF-induced junctional disruption and ROS

production similarly involves Rac1 activation [49]. Moreover, VEGF-induced Rac activation requires VEGFR-2, active Src, subsequent activation of the guanine nucleotide exchange factor Vav2, and finally activation of Rac1 [51]. This pathway was shown to directly affect the phosphorylation of VE-cadherin and β-catenin in a ROS dependent manner, thereby affecting adherens junction stability and endothelial permeability [52]. Therefore, production of ROS downstream of Rac in this pathway is one mechanism of inducing EC permeability, although other ROS-independent pathways have also been suggested [32, 53].

One of the mechanisms by which ROS could alter vascular permeability involves regulation of junctional protein phosphorylation, which as discussed earlier, is often associated with increased permeability. ROS can strongly inactivate protein phosphatases by oxidation of a critical cysteine residue in the catalytic site [54]. The prevalence of phosphatases (VE-PTP, DEP-1, PTPμ, SHP-2) at EC junctions suggests they are critical for maintaining the low basal phosphorylation levels conducive for junctional integrity [55]. Localized production of ROS and inactivation of phosphatases at these sites would therefore contribute to elevated phosphorylation and junctional disruption.

Regulation of Rho GTPases by ROS

ROS may also regulate junctional permeability by affecting the organization of the actin cytoskeleton [56]. This raises the intriguing possibility that ROS may regulate GTPase activity. A number of reports have shown indirectly that ROS can affect GTPase activity. The first major observation was made in by Nimnual et al., in which it was shown that ROS generation

downstream of active Rac1 (presumably through NADPH oxidase) results in decreased RhoA activity [57]. This “Bar-sagi pathway” involves ROS-mediated inactivation of a tyrosine phosphatase, leading to elevated p190 Rho GAP activity and therefore decreased RhoA activity. This observation that elevated Rac1 activity can lead to decreased RhoA activity has been made before, although not in the context of ROS [58]. Similar findings were made in the context of ROS by Wojciak-Stothard et al., who measured RhoA and Rac1 activities in pulmonary aortic endothelial cells under conditions of hypoxia [59]. Hypoxia was shown to induce a gradual decrease in Rac1 activity, and an increase in RhoA activity. Interestingly, hypoxia was also shown to result in decreased ROS production, thereby, potentially implicating ROS as an indirect mediator of GTPase activity. More recently, a novel activator of NADPH oxidase (Nox4) called Poldip2 was found to stimulate ROS production, RhoA activation, and stress fiber formation in vascular smooth muscle cells [60].

Although these studies do indicate that ROS could be a mediator of RhoA activity, it is critical to note that they do not show a direct effect of ROS on RhoA. There is considerable evidence that ROS are capable of directly regulating Rac1 and RhoA by cysteine oxidation. Work on the regulation of GTPases by ROS and RNS actually began with studies of Ras in which treatment of T-cells with nitric oxide gas resulted in GDP/GTP exchange and an increase in the pool of Ras-GTP [61]. It was later found that NO treatment caused an oxidation of cysteine 118 which was responsible for nucleotide exchange and subsequent Ras activation [62].

This work was later extended to include Rho GTPases, specifically Rac1 and RhoA. It was found that a subset of Rho GTPases contain a novel, conserved motif (GXXXXGK(S/T)C) that is critical for regulation by ROS and RNS [63]. In Rac1, this motif contains cysteine 18. RhoA is interesting in that two cysteines occur within the conserved motif (cysteine 16 and cysteine 20). This raises the intriguing possibility that oxidation could result in formation of an intramolecular disulfide bond, which could affect GTPase activity. The formation of a disulfide bond was in fact observed, when recombinant, purified RhoA protein was exposed to NO radical [64]. This led to decreased nucleotide exchange, which could be reversed by addition of reducing agent (GSH). Although high levels of oxidants could result in disulfide bond formation, it was hypothesized that low levels of ROS could result in activation of RhoA due to radical mediated exchange of GDP for GTP. The studies described in Chapter 2 are based on this hypothesis, that ROS generation in cells can directly affect RhoA activity.

Leukocyte TEM

The regulated transmigration of leukocytes across the endothelial lining of the vasculature is critical to the inflammatory response. However, when TEM is excessive or inappropriately localized, it can initiate many pathological processes. For example, the transmigration of leukocytes is an early step in the development of atherosclerotic plaques [65]. Also, the pathogenesis of multiple sclerosis involves a massive influx of leukocytes transmigrating into the brain,

leading to tissue damage and neurological disability [66]. Numerous chronic inflammatory diseases such as arthritis also involve TEM.

The process of leukocyte TEM is carefully coordinated by a large number of different adhesion molecules expressed on the surface of endothelial cells. Most adhesion molecules are expressed at low basal levels in resting endothelial cells, however, the inflammatory response causes activation of endothelial cells and subsequently increased expression of adhesion molecules. These adhesion molecules interact with ligands expressed on the surface of leukocytes. Although it is likely that each type of leukocyte utilizes unique mechanisms to mediate TEM it is generally accepted that leukocytes follow a general, stepwise process of adhesion and TEM.

Steps in TEM

Leukocyte adhesion and TEM occurs in sequential steps, each of which initiates signaling that facilitates progression to the next step [67, 68]. The process begins with the capture of leukocytes on ECs at sites of inflammation. This capture is largely mediated by weak interactions between selectins on the surface of endothelial cells and a variety of glycosylated ligands on the surface of leukocytes, such as CD44, PSGL-1, and ESL1. Interestingly, although the interaction between selectins and their ligands is relatively weak, a threshold level of shear stress is required for efficient binding [69, 70]. As leukocytes roll along the vessel wall, chemokines expressed by activated endothelial cells result in activation of integrins on the leukocyte surface. Importantly, cytokines regulate a positive feedback loop which promotes the production of more adhesion

molecules and cytokines. Activated integrins serve as ligands for the cell adhesion molecules, ICAM-1 and VCAM-1. MAC-1 and LFA-1 are ligands for ICAM-1, and VLA-4 is a ligand for VCAM-1 (described in detail below). Interestingly, it has been recently reported that E-selectin binding to its leukocyte ligand PSGL-1 promotes the activation of LFA-1, thereby promoting slow leukocyte rolling [71]. This is yet another example of how initial steps in the adhesion and transmigration process directly promote subsequent steps. Although ICAM-1 and VCAM-1 support rolling, they are best described in their role of mediating firm adhesion of leukocytes to the endothelial surface. Firm adhesion allows leukocytes to spread and begin to migrate along the vessel wall, prior to diapedesis. This process of intraluminal crawling is thought to be critical for efficient TEM, as it allows the leukocyte to find the "optimal" site to undergo diapedesis [72]. Interestingly, it has been found that ICAM-1 and one of its integrin ligands (MAC-1) are critical for this process [73].

When the leukocyte has reached an appropriate site, diapedesis can occur by either of two routes; the most prevalent mechanism *in vitro* is "paracellular" TEM, which requires transient junctional disruption as leukocytes migrate between adjacent cells. It appears that tricellular junctions may be preferential for paracellular transmigration due to their unique composition of discontinuous tight junctions and overlaying "flaps" [74]. Neutrophils seem to prefer these unique junctions *in vitro* under flow [75]. More recently, *in vivo* experiments have corroborated these *in vitro* findings and extended them to include a role for ICAM-1 enrichment at tricellular junctions [76]. TEM can also

occur by a “transcellular” route in which a leukocyte moves through the body of an EC, probably via the transient formation of a pore. Transcellular TEM has been reported *in vitro* and *in vivo* [77]. The factors which determine the “optimal” site for diapedesis and the route which is taken (paracellular vs transcellular) are still largely unknown. Common to both routes is the formation of a transmigratory “docking structure” which extends from the endothelium to surround the adherent leukocyte prior to the onset of diapedesis. The signaling mechanisms involved in formation of these structures and subsequently the completion of transmigration are discussed below.

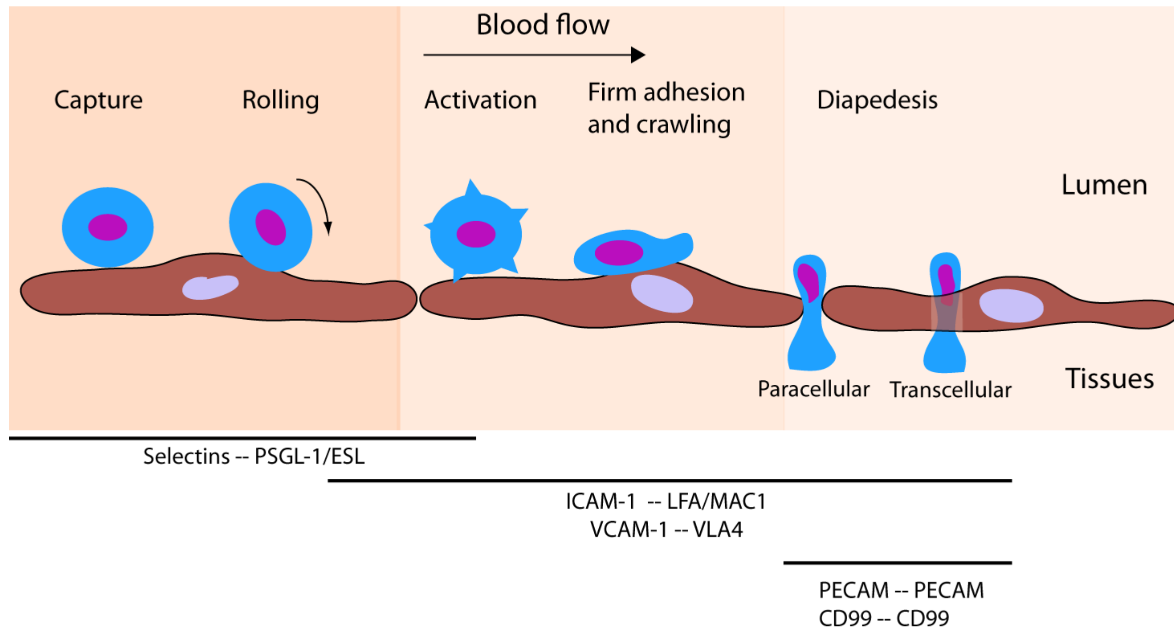


Figure 2. **Leukocyte adhesion and transmigration cascade.** Model illustrating the main steps in leukocyte TEM and the endothelial adhesion molecules and corresponding leukocyte ligands involved.

ICAM-1

Intercellular adhesion molecule 1 (ICAM-1) and vascular endothelial cell adhesion molecule 1 (VCAM-1) are particularly important for firm, integrin-mediated adhesion of leukocytes to ECs and subsequent TEM [78]. The role of these cell adhesion molecules (CAMs) has been extensively reviewed [79]. ICAM-1 is a member of the Ig-like superfamily of proteins, with 5 Ig like repeats in its extracellular domain and a short cytoplasmic tail [80]. In vitro, ICAM-1 is expressed constitutively on the surface of endothelial cells, albeit at low levels, however stimulation with IL-1B or TNF-alpha for 12-24 hrs can enhance surface expression by 40 times [81]. Interestingly, the increase in expression of ICAM-1 corresponds to downregulation of E-selectin expression. The importance of ICAM-1-mediated signaling to transendothelial migration is demonstrated by the deficient immune response exhibited by ICAM-1-null mice [82]. Furthermore, antibody blockade of ICAM-1 inhibits leukocyte TEM *in vivo* [83] and *in vitro*. Additionally, both the expression of ICAM-1 [84] and the activation of ICAM-signaling by crosslinking [82] have been shown to increase vascular permeability *in vitro*. The importance of ICAM's cytoplasmic tail has been demonstrated *in vitro*, in experiments using reconstituted cell lines. Sans et al. showed that expression of ICAM-1 in CHO cells, which don't express any endogenous cell adhesion molecules, significantly enhances neutrophil transmigration [85]. However, this effect was abolished when cells expressed a cytoplasmic tail-deleted mutant of ICAM-1. These results indicate that ICAM-1 alone can support

TEM and that this requires the interaction of ICAM-1's cytoplasmic tail with other proteins which are required for TEM.

VCAM-1

Similar to studies of ICAM-1, blocking VCAM signaling with function blocking antibodies against VCAM-1 or its integrin ligand VLA-4 inhibits leukocyte adhesion to ECs and decreases TEM, although not as significantly as blocking both ICAM1 and VCAM [86]. However, unlike ICAM, whose integrin ligands are expressed by all leukocytes including neutrophils, VCAM's integrin ligand (VLA-4) is not expressed on neutrophils [87].

Although VCAM may not contribute significantly to early stages of acute inflammation, which is mediated largely by neutrophils, it does play significant roles in chronic inflammation particularly atherosclerosis. Atherosclerosis is a chronic inflammatory condition, whose initial stages are mediated primarily by monocytes. This is reflected in observations that VCAM is the first adhesion molecule expressed prior to atherosclerotic plaque development [88]. *In vivo* models of carotid injury and atherosclerosis, have shown that anti-VCAM antibodies prevent monocyte adhesion [89] and that neointimal formation can be reduced by administration of anti-VLA4 antibodies [90] or siRNA knockdown of VCAM [91].

Signaling downstream of ICAM and VCAM

Figure 3 illustrates three potential mechanisms downstream of VCAM-1 and ICAM-1 engagement which lead to paracellular TEM of leukocytes: Rho GTPase signaling, ROS-mediated regulation, and tyrosine phosphorylation of

junctional components. These diverse signals, involving numerous pathways are discussed below.

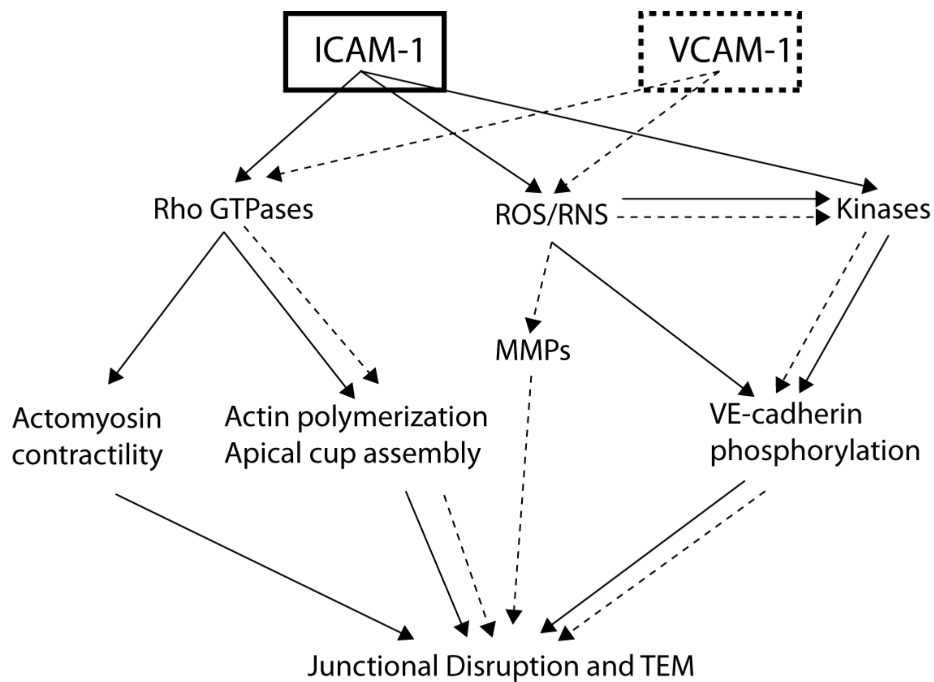


Figure 3. Signals from endothelial ICAM-1 and VCAM-1 which affect endothelial barrier function during TEM. Signals downstream of ICAM (solid lines) and VCAM-1 (dotted lines) are unique, but overlap extensively. A yet undetermined pathway from ROS/RNS to Rho GTPases is yet to be determined, and will be discussed in Chapter 3.

Rho GTPases

One well-studied outcome of ICAM-engagement is RhoA activation. Artificially simulating leukocyte adhesion by crosslinking ICAM-1 with antibodies results in stress fiber formation downstream of Rho GTP-loading [92, 93]. By using C3 transferase, a bacterially-derived Rho inhibitor, Adamson and colleagues showed that ICAM-1-stimulated cytoskeletal rearrangements required for efficient T-lymphocyte TEM were dependent on RhoA activation [94]. Treatment of ECs with Y-27632, an inhibitor of Rho kinase, or inhibitors of myosin light chain kinase also reduced neutrophil TEM [95, 96]. Our laboratory has recently shown that ICAM-1 engagement also activates RhoG [97]. This activation was critical for formation of the apical membrane protrusions which extend from ECs to partially engulf adherent leukocytes. These “docking structures” may facilitate the development of leukocyte pseudopods which extend through EC junctions [98]. Depletion of endothelial RhoG decreased cup formation and also inhibited leukocyte TEM. Further investigation revealed that the cytoplasmic tail of ICAM-1 binds SGEF, a RhoG guanine nucleotide exchange factor (GEF), which provides a mechanism of RhoG activation downstream of ICAM-1.

VCAM-1 has also been placed upstream of Rho family GTPases. Antibody-mediated VCAM-1 crosslinking on HUVECs results in activation of Rac1, induction of stress fibers, and formation of gaps in the EC monolayer leading to a decrease in electrical resistance [99]. The junctional disorganization induced by VCAM-1 engagement was dependent on ROS production. The role of

VCAM in production of ROS through Rac1 activation is discussed below. In addition to ICAM-1, VCAM-1 is also present in the cup-like docking structures which form at the site of leukocyte-EC interaction [100, 101]. However the role of VCAM in these structures has not been fully investigated.

Reactive Oxygen Species

ROS have been implicated as an important mechanism regulating leukocyte TEM. Endothelial ROS production upon adhesion of neutrophils has been shown to mediate cytoskeletal changes and cellular stiffening of endothelial cells to promote TEM [100]. Importantly, ICAM-1 blocking antibodies prevented ROS production and cytoskeletal changes induced by neutrophil adhesion, indicating that ICAM is upstream of ROS production. The source of ROS was identified as xanthine oxidase. P38 mitogen-activated protein kinase (MAPK) was later shown to be a downstream target of ROS in this system [101]. These studies were performed in pulmonary microvascular endothelial cells. Interestingly, similar experiments in pulmonary arterial cells did not yield similar results [102], indicating that there may be potential differences in signaling pathways between cells from microvasculature versus large vessel cells. Differences in cell culture models may also explain the findings of Martinelli et al. Using antibody crosslinking, they found that activation of ICAM-1 in immortalized rat brain endothelial cells stimulated nitric oxide synthase (eNOS), and not NADPH oxidase [103].

By activating Rac1, VCAM-1 crosslinking results in NADPH oxidase activation and ROS production in ECs [104]. Generation of low levels of ROS

upon VCAM-1 engagement is required for stereotypical actin cytoskeleton changes to occur. Extracellular release of ROS due to VCAM signaling results in the activation of EC-associated matrix metalloproteases (MMPs) [105], and this ROS-mediated activation of MMPs was required for efficient lymphocyte migration. This could potentially regulate TEM by MMP-mediated degradation of junctional components such as VE-cadherin. Evidence has been presented that ROS activates the redox-sensitive kinase Pyk2 [50]. Pyk2 phosphorylates β-catenin promoting its dissociation from VE-cadherin. Another kinase, PKC-α, was shown to be activated by oxidation downstream of VCAM. This activation was DAG-independent, and was required for efficient TEM of splenic lymphocytes [106]. Subsequently, the downstream target of PKC-α in this pathway was discovered to be protein tyrosine phosphatase 1B (PTP1B) [107]. The exact mechanism of how PTP1B activity promotes lymphocyte TEM remains to be determined; however this is yet another indication that a balance of phosphorylation is important for the maintenance of junctional integrity and that ROS may play a role in determining this balance.

Phagocytic cells generate high concentrations of extracellular ROS in a respiratory burst event mediated by the NADPH oxidase complex [108]. Exogenous ROS have been shown to enhance neutrophil binding to ECs due to increased expression of EC cell surface adhesion molecules [109]. Additionally, ROS released from fMLP-stimulated neutrophils was found to increase vascular permeability [110]. It is intriguing to consider that leukocyte-generated ROS may act locally on ECs to trigger intracellular signaling that facilitates TEM. This

mechanism of signaling may amplify existing signals initiated by EC-generated ROS or initiate separate pathways. One potential pathway for amplification of existing signals by ROS is stimulation of adhesion molecule expression. *In vitro* experiments have shown that treatment of endothelial cells with ROS, increases neutrophil adhesion due to enhanced ICAM-1 expression [111]. Similar results were obtained in vivo, in which animals were infused with hypoxanthine and xanthine oxidase as a superoxide generating system [112]. Intravital microscopy of mesenteric vessels showed that ROS promoted leukocyte adhesion and that this effect could be overcome by administration of antibodies against ICAM-1 integrin ligands.

Tyrosine phosphorylation of junctional proteins

VE-cadherin plays a critical role in regulating paracellular permeability during TEM. Adhesion of leukocytes to ECs has been shown to induce a localized, transient disruption of VE-cadherin which is required for efficient TEM [113]. Tyrosine phosphorylation of junctional components has been implicated as an important mechanism for regulating junctional integrity during TEM. For example, inhibition of protein tyrosine phosphatases in ECs increases tyrosine phosphorylation of AJ components and promotes neutrophil TEM [28, 114]. ICAM-1 engagement results in tyrosine phosphorylation of VE-cadherin through activation of Src and Pyk2 kinases [115]. Phosphorylation on tyrosines 658 and 731 inhibits binding of p120-catenin and β-catenin [30], and increases TEM of neutrophils [115]. Similarly, ICAM-mediated VE-cadherin phosphorylation in brain microvascular cells increased paracellular TEM of lymphocytes [116].

Interestingly, this pathway involving ICAM-1 activation leading to VE-cadherin phosphorylation has also been shown to require nitric oxide signaling [103]. This is yet another example of the important role of ROS/RNS in TEM.

Src activation due to ICAM signaling has been reported previously to induce tyrosine phosphorylation of a number of proteins including cortactin [117]. A pathway has been defined linking cortactin phosphorylation to TEM of neutrophils [118]. It was concluded that cortactin phosphorylation by Src serves to link ICAM-1 to the actin cytoskeleton, aiding ICAM-1 clustering at sites of leukocyte adhesion.

Chapter 2: Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif

Summary

Rho family GTPases are critical regulators of the cytoskeleton and affect cell migration, cell-cell adhesion, and cell-matrix adhesion. As with all GTPases, their activity is determined by their guanine nucleotide-bound state. Understanding how Rho proteins are activated and inactivated has largely focused on regulatory proteins such as guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). However, recent in vitro studies have indicated that GTPases may also be directly regulated by redox agents. We hypothesized that this redox-based mechanism occurs in cells and affects cytoskeletal dynamics, and in this report we conclude this is indeed a novel mechanism of regulating the GTPase RhoA. We show that RhoA can be directly activated by reactive oxygen species (ROS) in cells, and that this requires two critical cysteine residues located in a unique redox-sensitive motif within the phosphoryl binding loop.

First, we show that ROS can reversibly activate RhoA and induce stress fiber formation, a well characterized readout of RhoA activity. To determine the role of cysteine residues in this mechanism of regulation, we generated cysteine to alanine RhoA mutants. Mutation of these cysteines abolishes ROS-mediated activation and stress fiber formation, indicating that these residues are critical for

redox-regulation of RhoA. Importantly, these mutants maintain the ability to be activated by GEFs . Our findings identify a novel mechanism for the regulation of RhoA in cells by ROS, which is independent of classical regulatory proteins. This mechanism of regulation may be particularly relevant in pathological conditions where ROS are generated and the cellular redox-balance altered, such as in asthma and ischemia-reperfusion injury.

Introduction

Rho family GTPases serve as critical regulators of cell migration, cell-cell adhesion, and cell-matrix adhesion, by transmitting extracellular and intracellular signals to effectors that act on the cytoskeleton. The best characterized members of the Rho family of GTPases are RhoA, Rac1, and Cdc42; each of which is associated with unique phenotypes and functions [119-121]. As with all canonical GTPases, the activity of Rho GTPases is determined by their guanine nucleotide-bound state. Rho GTPases are activated by binding GTP, which causes a conformational change in the protein that greatly increases the affinity for downstream effector proteins. These effector proteins are components of signaling cascades which ultimately lead to modulation of cellular functions. Conversely, GDP-bound Rho GTPases are unable to bind effector proteins and are therefore inactive. The switching between “on” and “off” states is tightly controlled by regulatory proteins which interact with GTPases to regulate guanine nucleotide binding [33]. Guanine nucleotide exchange factors (GEFs) activate GTPases by promoting the dissociation of GDP to allow the binding of GTP, which is available in great excess over GDP levels in the cytoplasm. GTPase

activating proteins (GAPs) promote the hydrolysis of GTP to GDP, preventing GTPase interaction with downstream effectors. GDP-dissociation inhibitors (GDIs) maintain the inactive state of the GTPase by preventing GDP-dissociation and membrane association [122]. All of these regulatory proteins are themselves affected by diverse upstream signals which serve to activate or inactivate Rho GTPase signaling pathways.

Yet another mechanism for regulating GTPase activity has been proposed that involves the action of redox agents, specifically reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS have been historically considered pathological agents which can react with and damage many biological macromolecules including DNA, proteins and lipids. However, there has been an increasing recognition that ROS and RNS can also function in cell signaling pathways [123, 124] in particular, those pathways involving phosphatases [125]. Most cell types produce ROS, either as byproducts of normal metabolism or by specific enzyme complexes. A major source of ROS in cells is the NADPH oxidase complex, which can be activated by Rac1 [126-128]. This generation of ROS by Rac1 has been implicated in the redox-mediated regulation of RhoA activity. Nimnual et al. described a novel mechanism in which downregulation of RhoA occurs via redox-mediated inactivation of the phosphatase LMW-PTP and subsequent elevation of p190 RhoGAP activity [57].

As well as affecting regulatory protein function, another body of work shows that at least *in vitro*, oxidizing agents can also directly regulate the activity of certain GTPases. Lander et al. were the first to show this with Ras; NO

treatment of recombinant Ras increased the proportion of GTP-bound Ras and endogenous NO activated Ras in human T cells [61, 129]. Campbell's group went on to describe a radical-based mechanism for the stimulation of nucleotide exchange on Ras by NO [130]. Interestingly, Campbell's group also identified a distinct redox-active motif located in the phosphoryl-binding loop in another subset of GTPases, the Rho family (RhoA, Rac1, and Cdc42) [63]. They found that treatment of purified recombinant GTPase with superoxide anion radical or nitrogen dioxide radicals *in vitro* resulted in guanine nucleotide dissociation. The proposed mechanism involves electron transfer of a thiol radical intermediate to the guanine base, which disrupts interactions between the guanine nucleotide and the GTPase, resulting in guanine nucleotide dissociation. In the presence of radical quenching agents, the intermediates are reversed and guanine nucleotide association can occur. Under conditions where GTP is in excess, such as in the cell, the end result is exchange of GDP for GTP, thereby activating the GTPase. Based on these *in vitro* findings, we hypothesize that ROS can directly affect GTPase activity in cells by oxidative modification of the critical cysteine residues within the redox-active motif. This may be a novel mechanism of regulating GTPase signaling cascades, independent but parallel to classical regulation by GEFs and GAPs.

In this paper, we show that RhoA can be directly activated by ROS in cells and that ROS-mediated activation of RhoA can induce cytoskeletal rearrangement. By using cysteine to alanine mutants, we demonstrate that ROS-mediated activation of RhoA is dependent on cysteines 16 and 20. Our

findings indicate that ROS-mediated RhoA activation is another potential regulatory mechanism in cells that can affect cytoskeletal dynamics.

Results

ROS induces reversible activation of RhoA

Peroxides are short-lived, membrane permeable oxidants that are generated *in vivo* by enzymatic complexes and as byproducts of cellular metabolism. To determine if exogenous ROS affects the activity of cellular RhoA, we treated REF-52 fibroblasts with low doses (0.1 μM to 10 μM) of a stable analog of hydrogen peroxide, t-butyl hydroperoxide (peroxide), for 10 min under serum-free conditions. The doses of exogenous peroxide used are physiologically relevant, based on measurements of intracellular ROS production made by others [123, 131]. Using standard Rhotekin-RBD pulldown assays, we observed that RhoA was significantly activated by peroxide treatment, as shown in Figure 4A and the accompanying quantification. To determine whether this activation is reversible, we treated REF-52 fibroblasts with 0.1 μM peroxide for 10 min, followed by washout with media only (Figure 4B). 0.1 μM peroxide activates endogenous RhoA, but the activity returns to baseline levels within 15-30 min upon washout. This reversibility suggests that the reducing environment of the cytoplasm and presence of intracellular anti-oxidants rapidly quench the effects of a bolus dose of exogenous peroxide. To determine whether endogenous ROS can activate RhoA, we used antimycin A, a compound that inhibits electron transport at complex III of the mitochondrial respiratory chain, thereby inducing the production of superoxide and other ROS [132, 133] (Figure 4C). Treatment

of cells with antimycin A resulted in activation of RhoA (Figure 4D). This activation was prevented by the presence of N-acetyl cysteine (NAC), a free radical and ROS scavenger. Having confirmed that endogenous ROS exerts the same effect on RhoA as extracellular peroxide, for all remaining experiments we use peroxide instead of antimycin A. We also examined whether peroxide would activate Rac1 in cells. With REF-52 fibroblasts, low levels of activation were detected in some experiments, but this was variable. However, with both HeLa cells (Figure 9) and endothelial cells (data not shown), we routinely observe robust activation of Rac1 in response to exogenous peroxide at 0.1 μM to 10 μM concentrations.

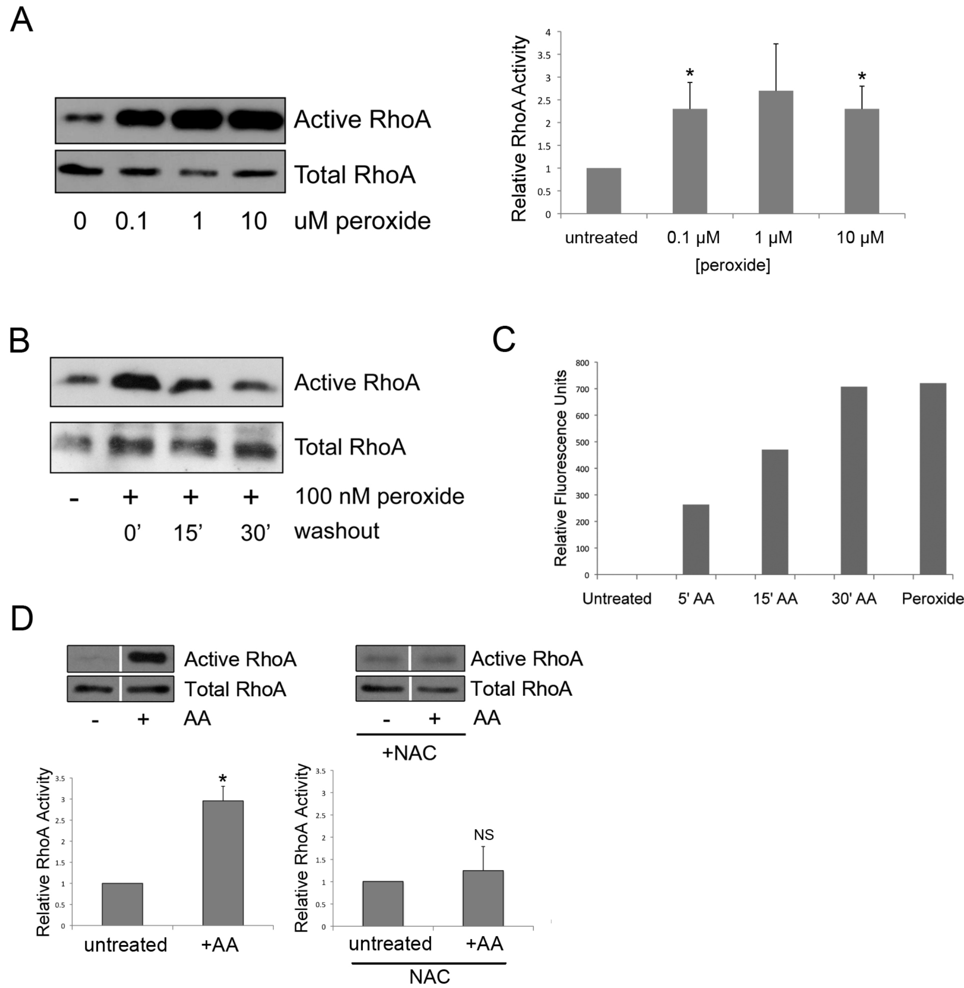


Figure 4. RhoA is reversibly activated upon exposure to ROS. (A) REF-52 fibroblasts were serum-starved followed by treatment with the indicated concentrations of t-butyl hydroperoxide (peroxide) in serum-free DMEM for 10 min. RhoA activity assays show that peroxide treatment results in the activation of endogenous RhoA. A representative blot of active RhoA sedimented by GST-RBD versus total RhoA from whole cell lysates is shown. ImageJ software was used to quantify RhoA activation. Relative RhoA activity is the ratio of active RhoA divided by total RhoA normalized to the untreated control. Graph represents the average +/- SEM of 5 independent experiments. * $p<0.05$ (B) REF-52 fibroblasts were serum-starved and treated as above with 100 nM peroxide for 10 min. Following peroxide treatment, the medium was either left unchanged (0') or replaced with serum-free DMEM for 15 or 30 min (15', 30'). The RhoA activity assay shows that peroxide-induced RhoA activation is reversible after washout. (C) Antimycin A induces ROS production. REF52 fibroblasts were loaded with 5 µM dichlorofluorescin diacetate (DCFDA) for 30 min under serum free conditions, followed by stimulation with 10 µM antimycin A (AA) for 5, 15 or 30 min. Stimulation with 10 µM peroxide for 5 min was used as a positive control. (D) REF-52 fibroblasts were serum-starved with or without 0.5 µM NAC followed by treatment with 10 µM antimycin A (AA) for 30 min. RhoA activity assays show that treatment with antimycin A results in the activation of endogenous RhoA. However, this activation is inhibited in cells pre-treated with NAC. Representative blots of active RhoA sedimented by GST-RBD versus total RhoA from whole cell lysates are shown. ImageJ software was used to quantify RhoA activation. Relative RhoA activity is the ratio of active RhoA divided by total RhoA normalized to the untreated control. Graph represents the average +/- SD of 3 independent experiments. * $p<0.05$, not signficiant (NS)

ROS treatment induces stress fiber formation.

To further explore what functional consequences this ROS-mediated RhoA activation might have in a cellular context, we looked at stress fiber formation as it is a well-characterized cellular readout of Rho activity [120]. For these experiments, Rat2 cells were used because upon serum-starvation they readily lose stress fibers, allowing a low baseline from which induction of stress fibers could be induced and more readily quantified. Rat2 cells were serum-starved for 1 h to cause loss of stress fibers, and then treated with either 5% FBS as a positive control, or 1 μM peroxide for 15 min. Cells were fixed and F-actin was detected with Texas-Red labeled phalloidin. As expected, serum starvation results in significant loss of actin stress fibers in Rat2 fibroblasts (Figure 5A). Importantly, peroxide treatment induced formation of prominent stress fibers to a similar extent as adding back serum (Figure 5A). Stress fiber induction was quantified by scoring cells for the presence of stress fibers and is represented as an index of stress fiber induction in Figure 5B. The induction of stress fibers by peroxide and FBS correlated with activation of RhoA in Rat2 cells (Figure 5C). To further show the role of RhoA in the induction of stress fibers by peroxide and FBS, we used the Rho kinase inhibitor Y27632. Y27632 inhibits Rho kinase and therefore Rho-mediated contractility, preventing the formation of stress fibers [132]. Pre-treatment with Y27632 significantly reduced the percent of cells with prominent stress fibers after peroxide or FBS treatment (Figure 5D). The ability of ROS to induce cytoskeletal alterations has been described previously, particularly in the context of oxidant-mediated changes in endothelial cell

permeability. These studies used both exogenous hydrogen peroxide [133-135], or superoxide and related molecules produced via the xanthine oxidase system [136]. It is important to note that these studies used peroxide concentrations in the range of 100-250 μM, and/or treatment for much longer time (1-6 hours) than we have utilized here (1 μM for 15 min). In addition to the formation of stress fibers, ROS treatment caused monolayer gap formation and increased permeability in these studies [134, 136], which is also indicative of elevated RhoA activity and increased actomyosin contractility induced via the Rho/Rho kinase signaling pathway. Consistent with these earlier reports, the data in Figure 4 and Figure 5 reveal that low concentrations of ROS activate RhoA and promote stress fiber formation in fibroblasts.

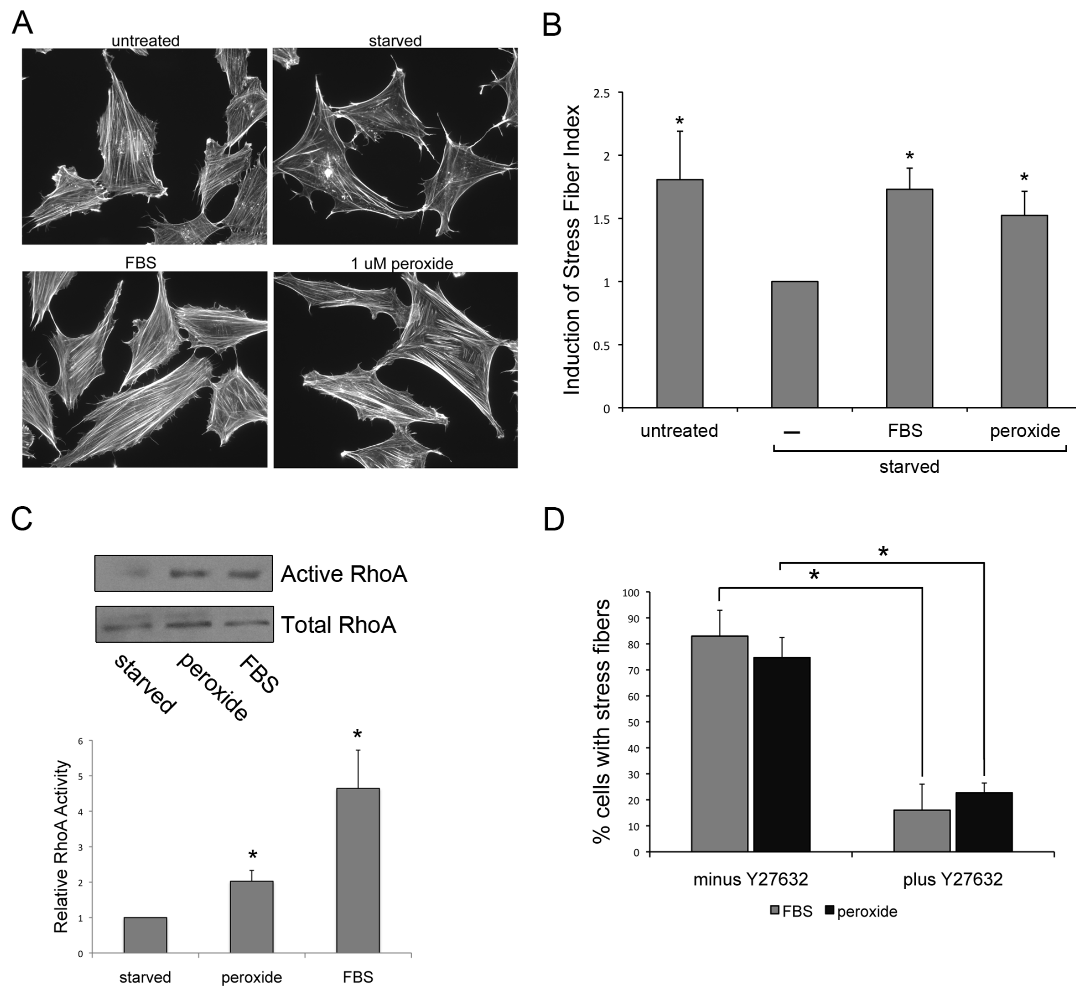


Figure 5. ROS treatment induces stress fiber formation. (A) Rat2 fibroblasts were grown on fibronectin-coated coverslips and either untreated, serum-starved for 1 h, or serum-starved followed by 15 min treatment with 5% FBS or 1 μM peroxide. F-actin staining was detected with Texas-Red labeled phalloidin. Serum-starvation results in loss of actin stress fibers; treatment with either FBS or peroxide induces prominent stress fibers. (B) Cells treated as above were scored for the presence of stress fibers and graphed as an index of stress fiber induction (normalized to serum-starved). Both FBS and peroxide result in an equally significant induction of stress fibers. Graph represents the mean of 3 independent experiments ± SD, with > 300 cells counted for each condition. Actual percent cells with stress fibers for each condition is 75.3 ± 7.5% (untreated), 42.3 ± 4.6% (starved), 64.3 ± 6.1% (serum-starved and peroxide treated), and 73 ± 6.4% (serum-starved and FBS treated). * $p < 0.05$ (C) Rat2 fibroblasts were serum-starved for 1 hr, followed by treatment with either 1 μM peroxide or 5% FBS for 15 min. RhoA activity assays show that both peroxide and FBS activate RhoA. A representative blot of active RhoA sedimented by GST-RBD versus total RhoA from whole cell lysates is shown. ImageJ software was used to quantify RhoA activation. Relative RhoA activity is the ratio of active RhoA divided by total RhoA normalized to the untreated control. Graph represents the average +/- SD of 2 independent experiments. * $p<0.05$ (D) Rat2 cells were grown as in panel A, serum-starved for 1 hr ± 5 μM Y27632, and then treated for 15 min with 1 μM peroxide or 5% FBS. F-actin staining was detected with Texas-Red labeled phalloidin. Pre-treatment with Y27632 significantly reduces the percent of cells with prominent stress fibers after both peroxide and FBS treatment. Graph represents the mean of 3 independent experiments ± SD with >300 cells counted for each condition.

Redox-sensitive cysteine residues are required for ROS-mediated activation of RhoA

What is the mechanism for this redox regulation of RhoA? Previously published *in vitro* observations have shown that RhoA has a redox-sensitive motif (GXXXCGK(S/T)C) containing two cysteine residues in the phosphoryl binding loop [63, 64]. As shown in Figure 6A, these cysteine residues are conserved in human, mouse, and rat RhoA. Previous *in vitro* studies revealed that oxidation of these cysteine residues, particularly cysteine 20, resulted in displacement of the bound nucleotide. Subsequent reduction allowed nucleotide to rebind, i.e. for nucleotide exchange to occur [63, 64]. To test whether these residues might play a role in the regulation of RhoA activity in cells by ROS, we mutated both cysteine 16 and 20 to alanine residues, thus making them resistant to oxidative modification. To prevent interference of endogenous RhoA in our experiments, we designed an adenovirally delivered miRNA construct targeted against a conserved region of RhoA, as shown in Figure 6B. Myc-tagged, non-targeted wildtype (wt) and C→A (C16/20A) mutants were designed with 5 silent mutations within the miRNA targeting sequence, thus allowing re-expression of the myc-tagged wt and C16/20A mutant proteins. Figure 6C demonstrates effective knockdown of endogenous RhoA and simultaneous re-expression of either wt or C16/20A myc-RhoA. Importantly, these myc-tagged, non-targeted RhoA constructs retain the ability to bind GTP as shown by GST-RBD pulldown assays in Figure 6D.

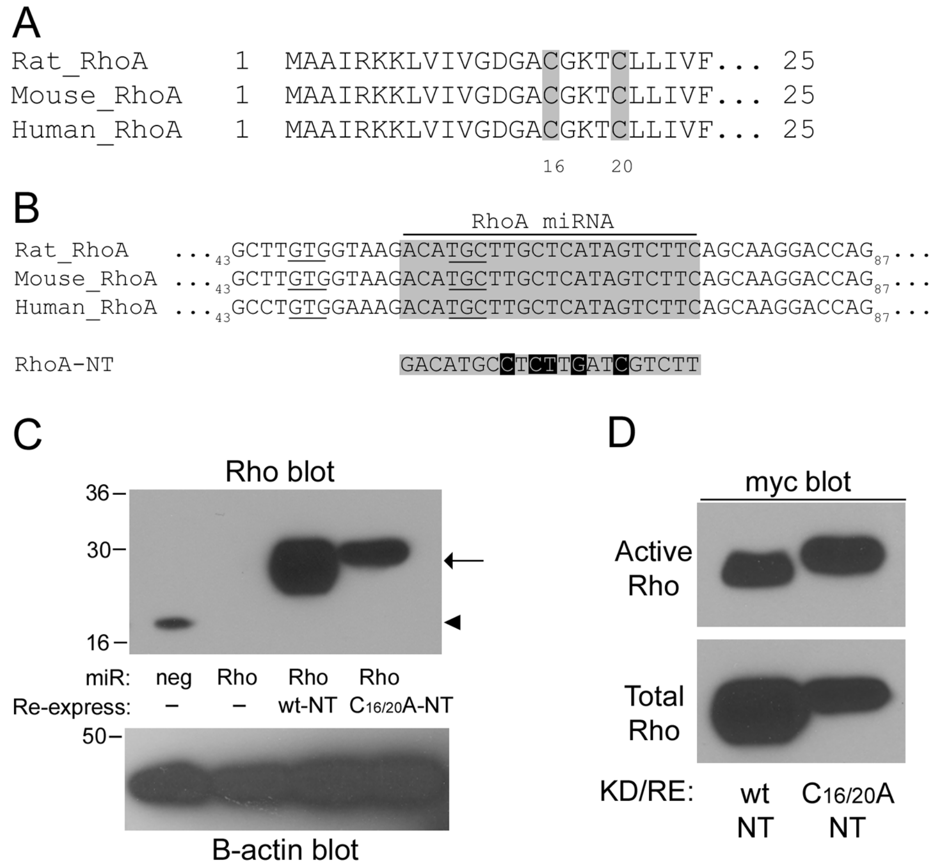


Figure 6: Characterization of RhoA miRNA and re-expression of non-targetable (NT) redox-resistant RhoA mutant constructs. (A) RhoA protein sequence (amino acids 1-25) showing conserved putative redox-sensitive cysteines C16 and C20 (shaded residues). (B) Nucleotide sequence (bp 43-87) of rat, mouse, and human RhoA showing the region targeted by the RhoA miRNA used in this study (shaded area). To re-express non-targeted RhoA constructs five silent mutations were introduced in the miRNA targeting region (black boxes). Nucleotides corresponding to C16 and C20 are underlined. (C) Knockdown of endogenous RhoA and simultaneous re-expression of non-targeted (NT) mutant constructs. REF-52 cells were infected with negative control or RhoA miRNA-encoding adenovirus together with virus encoding wt or C16/20A myc-RhoA where indicated. After 72 hours, total cell lysates were analyzed by western blot with a RhoA and B-actin antibody (loading control). Arrowhead indicates endogenous RhoA; arrow indicates the slower migrating non-targeting, myc-RhoA constructs. (D) Activity assay of wt and C16/20A myc-RhoA constructs. REF-52 cells were infected with negative control or RhoA miRNA-encoding adenovirus together with virus encoding wt or C16/20A myc-RhoA. After 72 hours, cells were processed for RhoA activity assays (in the presence of serum) and analyzed by western blot with a myc antibody.

To test whether cysteine 16 and 20 are required for the redox-sensitivity of RhoA, we examined the ability of C16/20A myc-RhoA to be activated by ROS in cells. REF52 cells expressing wt or C16/20A myc-RhoA were treated with various doses of peroxide under serum-free conditions, followed by RBD pulldown assay to detect RhoA activity. As shown in Figure 7A and the accompanying quantification (Figure 7B), while wt myc-RhoA responds to peroxide similarly to endogenous RhoA with activation over baseline levels, the C16/20A myc-RhoA mutant is resistant to activation by ROS. Importantly, data shown in Figure 7 indicate that while the C16/20A RhoA mutant is unresponsive to ROS treatment, it is still capable of being activated and inactivated by other physiological stimuli. Thrombin treatment is a well-characterized stimulus that induces RhoA activation in a wide variety of cell types [137, 138], an effect that is mediated through the action of p115 RhoGEF, among others [139-141]. Figure 7C shows that thrombin treatment activates wt RhoA as expected, and also rapidly activates C16/20A myc-RhoA, indicating that the mutant retains the ability to be activated by GEFs. Conversely, we tested the ability of C3 toxin to inactivate C16/20A myc-RhoA. C3 inactivates RhoA by ADP-ribosylation, and treatment of cells with this toxin results in a characteristic dendritic phenotype [142, 143]. REF52 cells expressing either wt or C16/20A myc-RhoA respond to C3 as indicated by this altered morphology (Figure 7D). Together, the results shown in Figure 7 indicate that the cysteine to alanine mutations confer resistance to ROS-mediated GTPase activation, but notably, still maintain responsiveness of the GTPase to other physiological stimuli.

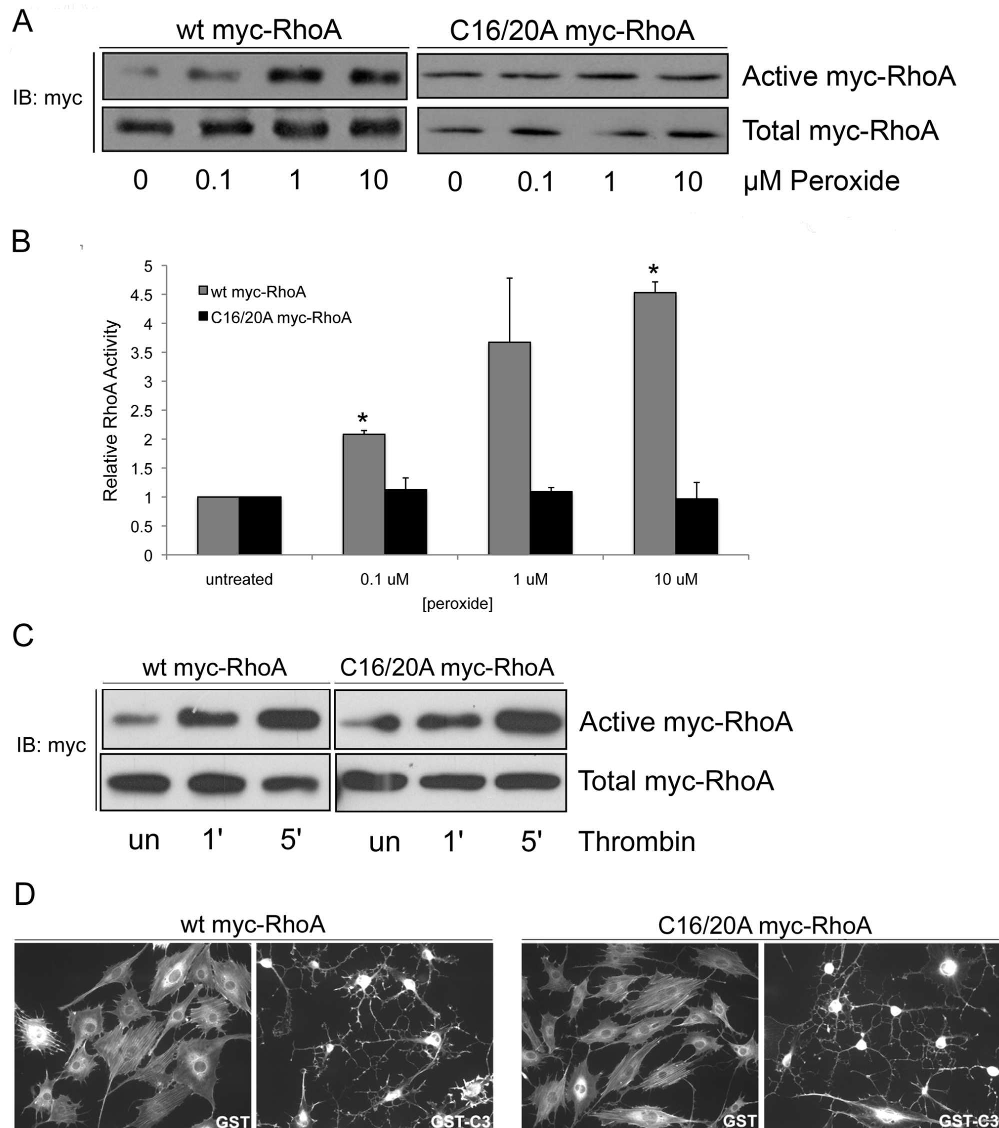


Figure 7. C16/20A myc-RhoA is not activated by ROS but responds normally to other physiological stimuli. (A) REF-52 cells expressing wt or C16/20A myc-RhoA were serum-starved and treated with the indicated concentrations of peroxide for 10 min. RhoA activity assays show that the activity of wt myc-RhoA increases in response to peroxide, however, the C16/20A mutant is resistant to activation by peroxide. (B) ImageJ software was used to quantify RhoA activation. Peroxide treated conditions are normalized to the corresponding untreated controls. Graph represents the average +/- SEM of 2 independent experiments. * $p<0.05$ compared to untreated control. (C) REF-52 cells in which endogenous RhoA had been knocked down and re-expressing wt or C16/20A myc-RhoA were serum starved and treated with 1 U/mL thrombin to test their responsiveness to GEF-mediated activation. RhoA activity assays show that both wt and C16/20A myc-RhoA are activated by thrombin at 1 and 5 min. (D) REF-52 cells in which endogenous RhoA had been knocked down and expressing wt or C16/20A myc-RhoA were treated with either GST alone or GST-C3, as described in the methods. F-actin staining was detected with Texas-Red labeled phalloidin. C3 treatment results in a characteristic dendritic morphology due to RhoA inactivation. Cells expressing either wt or C16/20A myc-RhoA develop the C3 phenotype.

ROS-mediated stress fiber induction requires redox-regulation of RhoA.

We went on to test how the C→A mutations affect the cellular function of RhoA by measuring peroxide-induced stress fiber induction in Rat2 cells as in Figure 8, but with cells re-expressing either wt or C16/20A myc-RhoA after knockdown of the endogenous protein. As shown in Figure 8, both wt and C16/20A myc-RhoA expressing cells have an equivalent amount of stress fibers in an unstarved state, and both lose stress fibers to a similar degree upon serum-starvation. However, while addition of 1 μM peroxide for 15 min induces stress fiber formation in cells re-expressing wt myc-RhoA cells expressing C16/20A myc-RhoA do not show any significant induction of stress fibers. These results confirm that cysteine 16 and/or 20 are specifically required for ROS-induced stress fiber formation by RhoA.

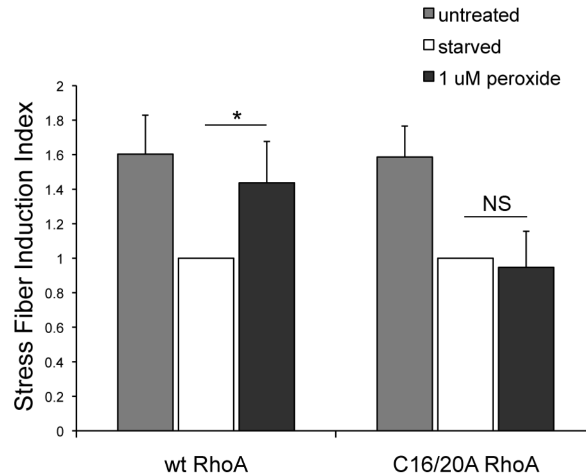


Figure 8. ROS induction of stress fibers is dependent on redox-regulation of RhoA. Rat2 fibroblasts were infected with adenoviruses to knockdown endogenous RhoA and re-express wt or C16/20A myc-RhoA. These cells were seeded on fibronectin-coated coverslips, and either left unstarved (DMEM + 5% FBS), or serum-starved for 1 h followed by treatment with DMEM alone, or 1 μM peroxide in DMEM for 15 min. Quantitation of stress fiber induction shows that compared with the starved condition, peroxide treatment induces a statistically significant increase in stress fibers in cells expressing wt myc-RhoA but not in cells expressing redox-resistant C16/20A myc-RhoA. Graph represents the mean of 3 independent experiments, with > 300 cells counted for each condition (error bars equal SD). * $p<0.05$; NS, not significant. Actual percent cells with stress fibers for each condition is: (unstarved) wt myc-RhoA 76.7 ± 5.0%, C16/20A myc-RhoA 78.1 ± 5.2%; (starved) wt myc-RhoA 48.3 ± 5.7%, C16/20A myc-RhoA 49.6 ± 5.0%; (peroxide) wt myc-RhoA 68.5 ± 4.9%, C16/20A myc-RhoA 46.3 ± 7.4%.

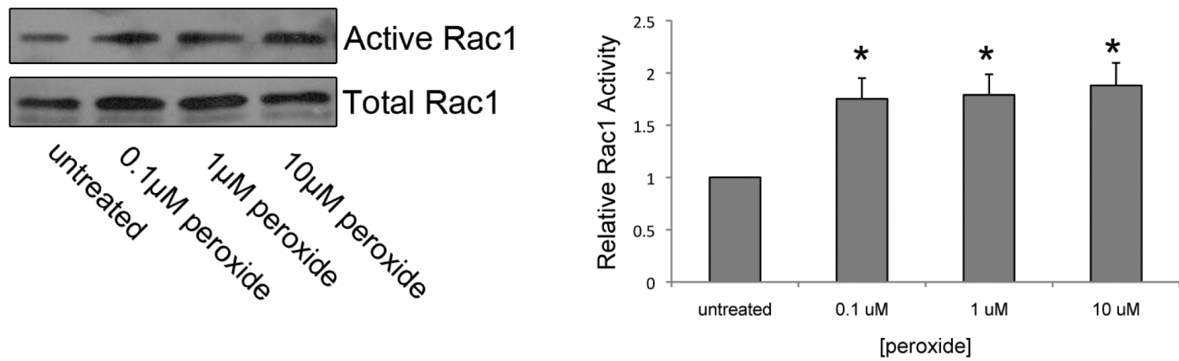


Figure 9. Rac1 activation by peroxide. Peroxide activates Rac1 in Hela cells. HeLa cells were serum-starved and treated with the indicated concentrations of peroxide for 10 min. Rac1 activity assays show that peroxide treatment results in activation of endogenous Rac1. A representative blot of active Rac1 sedimented by GST-PBD versus total Rac1 from whole cell lysates is shown. ImageJ software was used to quantify Rac1 activation. Graph represents the average +/- SD of 2 independent experiments. * $p < .05$ versus untreated control.

Discussion

ROS and RNS have been implicated in a variety of cell signaling pathways, including growth factor signaling [123, 124], inflammation [144], engagement of integrins [145, 146], and adhesion to extracellular matrix (reviewed in [147]). Hydrogen peroxide in particular has gained considerable attention as a potent signaling molecule [47]. Hydrogen peroxide and other ROS have been shown to be generated both intracellularly and extracellularly. Extracellular ROS generated by neutrophils can act on endothelial cells to affect vascular permeability [110] and EGF receptor engagement has been shown to induce the production of extracellular peroxide which can then permeate the cell membrane to affect intracellular signaling [148, 149]. Most recently, the role of paracrine signaling by externally-produced peroxide has been shown in a zebrafish wound healing model. Niethammer et al. show that injured epithelial cells secrete peroxide (in the range of 0.5 to 50 uM) to induce leukocyte recruitment to the wound [150].

There is strong evidence that Rac1-derived ROS can lead to oxidative inactivation of LMW-PTP, resulting in phosphorylation and activation of p190RhoGAP and subsequent downregulation of RhoA [57]. However, several studies have demonstrated that exogenous peroxide induces effects consistent with activation of RhoA [133-135, 151]. Furthermore, Campbell et al. demonstrated *in vitro* that ROS and RNS induce nucleotide displacement by direct cysteine oxidation in the phosphoryl-binding site of RhoA. If this oxidative event is reversed by reduction and GTP is present in excess, nucleotide

exchange (GTP binding) is favored and the GTPase is activated [64]. Notably, this occurs in the absence of GEFs. Our data support the mechanism described from their *in vitro* observations. We show that the activation of RhoA by peroxide is prevented by cysteine to alanine mutation of residues 16 and 20, although this mutant RhoA behaves normally in response to GEF-mediated situations (thrombin stimulation).

It is interesting that Zuckerbraun et al. found that treatment of cells with NO inhibits RhoA activity [152]. This apparent discrepancy has been observed in cell-based studies of the Ras GTPase, where both activation and inactivation of Ras have been observed in the presence of exogenously supplied RNS [153]. Redox regulation of Ras and Rho GTPase activity is likely to be dependent on a number of factors, including the type and level of cellular oxidants, reduction potential, as well as differences in cell lines resulting from variations in redox enzymes. If the oxidizing agent is in excess, or in the absence of a cellular reducing agent such as GSH, loss of GDP from RhoA can result in intramolecular disulfide formation between C16 and C20, and this form of RhoA is inactive, as it cannot bind nucleotide or interact with the GEF, Vav2, in *in vitro* studies [64]. However, under conditions where ROS/RNS are present at physiological levels and the reduction potential is high, as is the case within the cell cytoplasm, GTPase activation is expected, which is what we observe under our experimental conditions.

The cell's redox environment may also play a role in distinguishing regulation of RhoA by ROS as opposed to classical regulation by GEFs and

GAPs. It is likely that ROS-mediated pathways are occurring parallel to pathways involving classical regulatory proteins. However, since ROS are typically very reactive and have short half-lives, direct regulation of RhoA by ROS is likely to be more spatially and temporally confined. This confinement may be relaxed under conditions where the cell's redox environment is out of balance. Certainly, there are situations where ROS are generated at increased levels physiologically and pathologically. For example, the respiratory system is normally exposed to greater concentrations of ROS than other tissues. Pulmonary diseases associated with airway hyperresponsiveness, such as asthma and COPD, have been shown to be associated with increased ROS production [154]. ROS and RhoA activation have been attributed to tracheal smooth muscle contractility, which is a hallmark of these disease states [155, 156]. We suspect that increased levels of ROS under these conditions may directly activate RhoA to drive smooth muscle contractility. Similarly, ischemia-reperfusion injury is associated with increased ROS production, which leads to pathology of the vascular system [157]. ROS-mediated activation of RhoA has been demonstrated in vascular smooth muscle [158]. Based on our results, we would predict that ROS may activate RhoA signaling in smooth muscle and in the endothelium, contributing to the massive increases in vascular permeability associated with ischemia-reperfusion injury.

In addition, we suspect that other Rho family GTPases may be directly regulated by ROS given the work of Campbell et al. [63] . For example, we show that Rac1 activity is regulated by peroxide in epithelial and endothelial cells

(Figure 9 and data not shown). The regulation of Rac1 by ROS is particularly interesting because of its involvement in ROS production, through the NADPH oxidase complex [128], and its regulation by superoxide dismutase-1 (SOD1) [159]. SOD1 modulates intracellular ROS by converting superoxide to hydrogen peroxide. This creates a potential feedback loop in which Rac1 activity generates ROS which can regulate Rac1 activity through the redox-dependent sensor SOD1. Overall, these effects may be cell type specific, as the localization of a particular GTPase and baseline levels of activity likely play an important role in how that GTPase responds to ROS in terms of activity and phenotypic response.

We have shown that ROS can regulate RhoA activity in cells via a mechanism involving critical cysteine residues present in a redox-sensitive motif. This ROS-based mechanism of regulation was previously suggested by biochemical studies *in vitro* [64]. Our data support this occurring within cells and furthermore show that in a cellular context oxidative modification of these residues is a novel mechanism that can affect cytoskeletal dynamics.

Materials and Methods

Chemicals

T-butyl peroxide (peroxide) and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Antimycin A was purchased from Axxora (San Diego, CA). Y-27632 was purchased from Calbiochem/EMD Chemicals (Gibbstown, NJ). 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate

di(acetoxymethyl ester) (DCFDA) was purchased from Molecular Probes (Eugene, OR).

Cell Lines

REF52 [160], Rat2 fibroblasts (CRL-1764, American Type Culture Collection), and HeLa cells (CCL-2, American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma) and antibiotic-antimycotic solution (Gibco).

Knockdown of RhoA using miRNA adenovirus

miRNA adenoviral constructs were designed and engineered using the BLOCK-iT ™ Pol II miR RNAi expression vector system (Invitrogen) according to the manufacturer's protocol (Invitrogen). Briefly, double-stranded oligonucleotides were designed using Invitrogen's RNAi Designer (www.invitrogen.com/rnai) to form an engineered pre-miRNA sequence structure that targets a conserved region in human, mouse, and rat RhoA:

5'TGCTG<u>AAGACTATGAGCAAGCATGTC</u>GTTTTGGCCACTGACTGACGACATGCTCTCATAGTCTT3' (21 bp antisense target sequence underlined). Synthesized oligonucleotides were annealed and ligated into pcDNA 6.2-GW/EmGFP-miR. The EmGFP-miRNA cassette was subsequently shuttled through pDONR221(Invitrogen) by Gateway BP recombination and then into pAd-CMV-Dest Gateway vector by LR recombination. Each construct was sequence verified and virus was produced in 293A packaging cell line with the ViraPower Adenoviral Expression System (Invitrogen) using the manufacturer's recommended protocol.

Generation of non-targeting RhoA constructs and adenoviral-mediated expression

In order to simultaneously knockdown endogenous RhoA while allowing re-expression of RhoA mutants, non-targeting RhoA constructs were designed. Human RhoA in pCMV-myc was used as a template for site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis kit; Stratagene), wherein 5 silent DNA basepair mutations were made within the miRNA targeting region of RhoA. This non-targeted form of RhoA was then used as a template for site-directed mutagenesis in order to generate cysteine to alanine mutants at C16 and C20 (C16/20A myc-RhoA). Adenovirus was generated using the Virapower adenoviral expression system (Invitrogen) following the manufacturer's protocol as described above. For experiments, cells were infected by overnight incubation with adenovirus-containing media followed by addition of fresh DMEM/10% FBS for another 48 h.

Immunohistochemistry and measurement of stress fiber induction

Rat2 cells treated with adenovirus to knockdown endogenous RhoA while re-expressing either wt myc-RhoA or C16/20A myc-RhoA were plated onto fibronectin-coated coverslips (15 ug/ml) overnight, followed by serum-starvation for 1 h. Cells were treated for 15 min with either serum-free or 1 μM peroxide-containing DMEM, or 5% FBS-containing DMEM (positive control) before fixing/permeabilizing in 3.7% paraformaldehyde, then 0.02% Triton X-100. In some experiments, Rat2 cells were starved in the presence of 5 μM Y27632. Texas Red phalloidin (Molecular Probes) was used to stain F-actin. Immunofluorescence images were taken through a 20x objective (Zeiss plan-

Apochromat 20x/0.8) with a Zeiss axiovert 200M microscope equipped with a Hamamatsu ORCA-ERAG digital camera and acquired using Metamorph Workstation (Universal Imaging Corp.). To quantify stress fiber induction, GFP-positive cells were scored by a blinded observer for the presence or absence of stress fibers; the criteria were: organized, thickened parallel actin bundles throughout the majority of the cytoplasm. Results are plotted as a "stress fiber induction index" which was obtained by normalizing each treatment to the starved condition. Statistical significance was determined by Student's t-test of the average from 3 independent experiments.

RhoA activity assays

RhoA activity assays [161] were performed as described previously with minor modifications [162]. Cells were serum-starved in DMEM for 2 hours prior to performing assays to lower baseline RhoA activity and remove serum proteins which would interfere with experimental conditions. Cells were washed with ice-cold HEPES buffered saline (pH 7.4) and lysed in Buffer A (50 mM Tris pH 7.6, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 mM $MgCl_2$, 100 μM orthovanadate and protease inhibitors). Lysates were clarified by centrifugation and equalized for total volume and protein concentration. After incubation with glutathione S-transferase (GST)-Rho binding domain (RBD) beads, washing three times with ice-cold Buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$, 100 μM orthovanadate, with protease inhibitors, the bound fraction (active GTP-RhoA) was analyzed by SDS-PAGE. Total RhoA levels were similarly analyzed using a reserved aliquot of whole cell

lysate. Active RhoA and total RhoA were analyzed by Western blotting with an anti-RhoA antibody (monoclonal antibody 26C4; Santa Cruz Biotechnology, Santa Cruz, CA). The results were quantified using ImageJ software (NIH; Bethesda, MD). The relative amount of active RhoA was determined by taking the ratio of RhoA sedimented by GST-RBD beads (active RhoA) divided by the amount of total RhoA in the whole cell lysate. Statistical significance was determined using Student t-test (one-tailed).

Rac1 activity assays

Rac1 activity assays [58, 163] were performed as described previously with minor modifications [164]. Cells were serum-starved in DMEM for 1 hour prior to performing assays to lower baseline Rac1 activity and to remove serum proteins which would interfere with experimental treatments. Cells were washed with ice-cold HEPES buffered saline (pH 7.4) and lysed in Buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$, 100 μM orthovanadate, and protease inhibitors). Lysates were clarified by centrifugation and equalized for total volume and protein concentration. After incubation with glutathione S-transferase (GST)-PAK binding domain (PBD) beads and washing three times with ice-cold Buffer B, the bound fraction (active GTP-Rac1) was analyzed by SDS-PAGE. Total Rac1 levels were similarly analyzed using a reserved aliquot of whole cell lysate. Active Rac1 and total Rac1 were analyzed by Western blotting with a monoclonal anti-Rac1 antibody clone 102 (BD Transduction Laboratories, San Jose, CA). The results were quantified using ImageJ software (NIH; Bethesda, MD). The relative amount of active Rac1 was determined by

taking the ratio of Rac1 sedimented by GST-PBD beads (active Rac1) divided by the amount of total Rac1 in the whole cell lysate. Statistical significance was determined using Student t-test (one-tailed).

Measurement of ROS Generation

Formation of ROS was monitored by the conversion of non-fluorescent 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester) to fluorescent DCF. Cells were loaded with 5 μM DCF in serum-free DMEM for 30 min at 37 °C. After loading, cells were washed twice with phosphate-buffered saline, and incubated for an additional 20 min at 37 °C to allow for dye de-esterification. Cells were stimulated with antimycin A or peroxide as described in the figure legends. Fluorescence was determined using a fluorometer with an excitation of 485 and an emission of 520.

C3 treatment

GST-tagged recombinant proteins (either GST alone or GST-C3) were isolated from BL-21 bacteria using the manufacturer's protocol for GST fusion proteins (Amersham Pharmacia Biotech). Following adenovirus infection of REF-52 cells to knockdown endogenous and re-express wt or C16/20A myc-RhoA as described above, C3 toxin was introduced into these cells on coverslipsusing Lipofectamine according to the protocol of Maddox et al. [165]. After 3 hours of C3 exposure, cells were fixed and imaged as described above.

Chapter 3: Rho GTPases and ROS in regulating vascular permeability and TEM

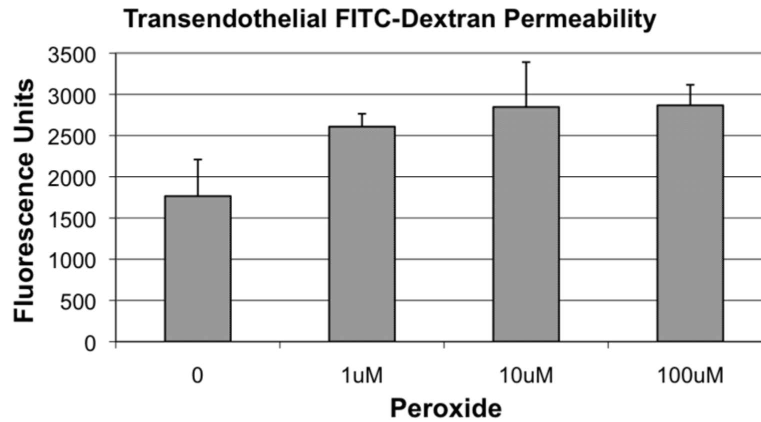
In this chapter, I will present and discuss a series of preliminary experiments in which I have examined the potential for GTPase regulation by ROS in endothelial cells. As discussed in Chapter 1, there is a significant body of work investigating Rho GTPases and ROS individually as critical regulators of endothelial cell-cell junctions and the processes which ultimately lead to leukocyte TEM. Many of these studies provide indirect evidence that Rho GTPases and ROS are connected, paving the way for my hypothesis that ROS can regulate Rho GTPase activity in endothelial cells and therefore impact permeability and TEM.

ROS promote vascular permeability *in vitro* and *in vivo*

Before specifically examining the role of ROS-mediated GTPase regulation, I will present preliminary evidence that ROS can regulate vascular permeability both *in vitro* and *in vivo*. This has been shown by others, but it was important for me to recapitulate the findings of others in my own experiments. Figure 10 shows the response of human umbilical vein endothelial cells (HUVECs) to low doses of exogenous hydrogen peroxide.

Measurement of transendothelial FITC-dextran flux shows increased permeability upon exposure to ROS over 30 minutes (figure 10A). Immunofluorescence studies by Erika Wittchen, shown in figure 10B, show disruption of VE-cadherin, a marker of EC junctions (arrowheads) after 15 minutes of treatment with 100nM hydrogen peroxide. Together these results indicate that ROS significantly affect endothelial cells and reduce endothelial barrier function .

A



B

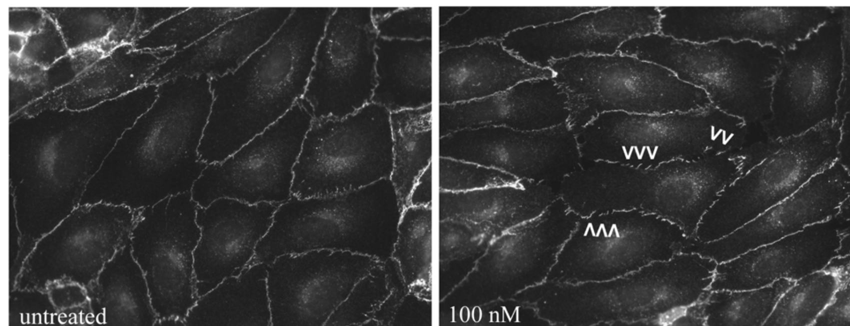
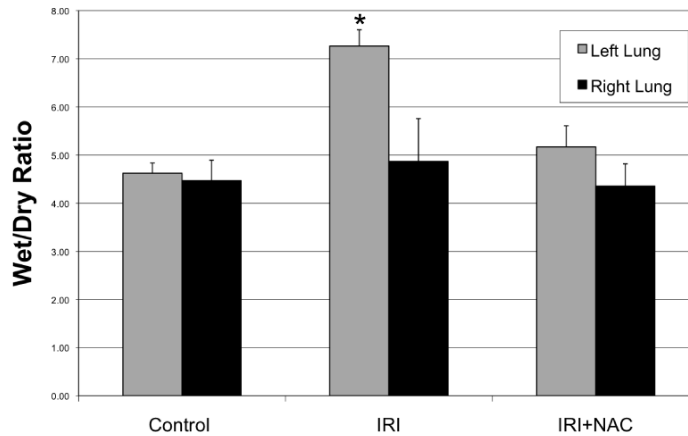


Figure 10. Peroxide treatment increases permeability of HUVEC monolayers. A. HUVECs were grown to confluence on matrigel coated transwell filters. Cells were treated with the indicated concentrations of hydrogen peroxide, and FITC-dextran was added to the upper chamber of the transwell. After 1 hr, media in the lower chamber was collected and fluorescence measured with a fluorometer. B. HUVECs were grown to confluence on matrigel coated glass coverslips. Cells were left either untreated or treated with 100nM hydrogen peroxide for 15 minutes, and fixed with 3.7% formaldehyde solution. Coverslips were stained for the junctional marker VE-cadherin. As indicated by the arrowheads, peroxide treatment results in gaps in the endothelial junctions.

In collaboration with Tom Egan, a cardiothoracic surgeon here at UNC, we also examined the contribution of ROS to mediating vascular permeability *in vivo* using a mouse model of pulmonary ischemia reperfusion injury (IRI). IRI is a pathological consequence of restoring blood flow to ischemic tissues and results in damage to the endothelium and massive increases in vascular permeability. Much of the pathology associated with IRI has been attributed to ROS production, as will be discussed in chapter 4. To simulate pulmonary IRI, the hilum of the left lung of a mouse was clamped for 1 hour followed by reperfusion for 30 minutes. The left and right lungs were removed, and the wet mass to dry mass ratio of the tissue used as an indicator of tissue fluid retention. A larger wet/dry ratio is indicative of increased fluid retention and permeability. For each mouse, the right lung which was not clamped serves as an internal control. Figure 11 shows that IRI of the left lung significantly increased wet/dry ratio, without affecting the permeability of the sham control right lung. However, when mice were pretreated with the ROS scavenger N-acetylcysteine (NAC), wet/dry ratios for the IRI left lung remained unchanged from control levels. This indicates the significant contribution ROS makes to changes in vascular permeability in IRI, and potentially in inflammation in general. Similar results have been published by others [166]. Interestingly, RhoA activity is also increased in lungs which have undergone IRI surgery (Figure 11B). This corroborates our hypothesis that ROS generation, for example during IRI, can stimulate RhoA activation *in vivo*.

A



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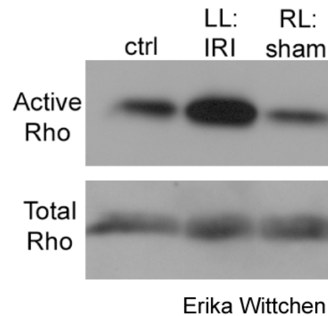


Figure 11. Scavenging ROS attenuates increased vascular permeability during IRI. A. Mice were subjected to IRI surgery as described in the text, and the lung tissue removed and weighed before and after drying. The wet/dry mass ratio serves as an indicator of edema and vascular permeability. IRI surgery on the left lung results in a significant increase in wet/dry ratio, whereas the sham control right lung is unaffected. *$p < .05$. Pre-treatment of the animals with the ROS scavenger N-acetylcysteine (NAC) prevents the increase in vascular permeability. Animals pre-treated with saline serve as controls. B. Mouse lung tissue was frozen in liquid nitrogen after IRI surgery, and used in RBD pulldown assays. IRI surgery of the left lung results in RhoA activation whereas RhoA activity in the sham control right lung is unchanged from control mice lung.

Depletion of ROS attenuates TEM

As discussed in Chapter 1, there is significant evidence that ROS are also involved in the processes leading to leukocyte adhesion and diapedesis. Both ICAM-1 and VCAM-1 have been placed upstream of ROS production in endothelial cells. To determine the contribution of ROS to leukocyte TEM, I measured the transmigration of primary human monocytes across HUVEC monolayers using phase video microscopy. Monocyte transmigration events were counted over 30 minutes, and are presented in figure 12 as the percentage of monocytes which underwent diapedesis. In this preliminary experiment, I found that pre-treatment of HUVECs with apocynin (an NADPH oxidase inhibitor) or allopurinol (a xanthine oxidase inhibitor) both reduced TEM. These results correlate with the findings of others. For example, Matheny et al. show that apocynin and DPI (both NADPH oxidase inhibitors) inhibit TEM of mouse splenic lymphocytes across monolayers of a mouse endothelial cell line [2]. As discussed earlier, leukocytes may also be an important source of ROS during adhesion and transmigration. This has been shown in the context of leukocyte transepithelial migration [167].

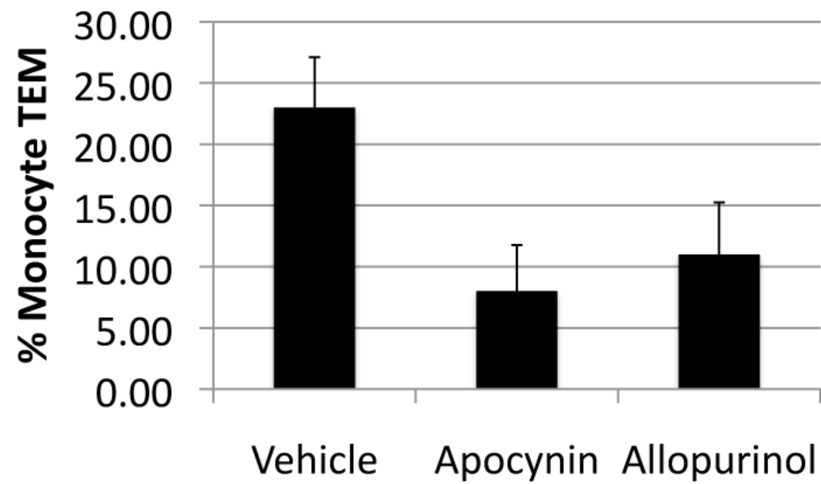


Figure 12. Monocyte transmigration is inhibited by treatment of HUVECs with apocynin or allopurinol. HUVECs were grown to confluence on matrigel coated coverslip bottom dishes, and treated with 10ng/ml TNF-alpha overnight. Primary human monocytes were isolated and allowed to adhere to HUVECs prior to beginning video microscopy. HUVECs were pre-treated with water (vehicle), apocynin, or allopurinol for 30 minutes prior to addition of monocytes. Transmigration events were counted over a period of 30 minutes. Pre-treatment of HUVECs with either apocynin or allopurinol inhibits monocyte transmigration.

ICAM-1 crosslinking results in stress fiber formation and activation of RhoA and Rac1

There is a significant amount of literature to support the hypothesis that leukocyte adhesion to endothelial cells results in ROS or RNS production, and that this could be a potential mechanism of regulating GTPase activity. As discussed in chapter 1, engagement of ICAM-1 and VCAM-1 on endothelial cells by antibody crosslinking or leukocytes has been shown to stimulate endothelial ROS or RNS production. In addition, there is a significant body of work describing RhoA or Rac1 activation downstream of ICAM-1 or VCAM-1 crosslinking. These papers are summarized in Table 1 below. As can be seen from the table, although the methods vary there is a common finding of stress fiber formation and either RhoA or Rac1 activation. For example, Etienne et al. show that ICAM-1 crosslinking for 10 minutes induces RhoA activation as measured by binding of p32-labeled nucleotide [92]. Crosslinking for longer timepoints (30 and 60 minutes) has also been shown to result in RhoA activation [168], whereas others have shown that RhoA activation disappears after 30 minutes of crosslinking with anti-ICAM1 coated beads [97]. Similar findings have been published for VCAM-1. For example, Cook-Mills et al. show Rac1 activation shortly after VCAM-1 crosslinking (30s – 2 min) which is gone by 12 minutes [104]. Van Wetering et al. show Rac1 activation by VCAM-1 crosslinking from 5-15 minutes, and show that stress fibers and gaps form in the endothelial monolayer after 30 minutes. Interestingly, this effect is diminished in C3-pretreated cells, indicating a role for RhoA downstream of VCAM-1. In addition to experiments utilizing antibody crosslinking to mimic adhesion molecule

clustering, there are examples of leukocyte adhesion stimulating GTPase activation. For example, Ishibashi et al. observed RhoA activation in primary human coronary microvascular ECs after 15-60 minutes of primary human monocyte adhesion [169]. They did not observe Rac1 activation. However, Ramirez et al. observed significant RhoA and Rac1 activation in primary brain microvascular ECs after 15 minutes of primary human monocyte adhesion [170].

	Method	Cell type	GTPase Activation?	Stress fiber/ Gap formation?
Etienne et al., J Immunol 1998 [92]	Crosslink ICAM-1 with soluble antibodies	Immortalized rat brain ECs (RBE4)	YES 10' XL ICAM-1: RhoA activation	N/D
Wojciak-Stothard et al., J Cell Biol 1999 [93]	Crosslink ICAM-1 with soluble antibodies	HUVEC	N/D	YES – stress fibers 30' XL ICAM-1
Thompson et al., J Immunol 2002. [168]	Crosslink ICAM-1 with soluble antibodies	HUVEC	YES 30' and 60' XL ICAM-1: RhoA Activation *observe a drop in RhoA activity with primary anti-ICAM antibody alone.	YES – stress fibers 60' XL ICAM-1
Van Wetering et al., Am J Physiol Cell Physiol 2003. [99]	Crosslink VCAM-1 with soluble antibodies	HUVEC	YES 5', 10', 15' XL VCAM-1: Rac1 activation	YES – stress fibers and gaps 30' VCAM-1 XL
Cook-Mills et al., Biochem J 2004. [104]	Crosslinking VCAM-1 with soluble antibodies	Immortalized mouse endothelial cell line (mHEVa)	YES 30s, 2' XL VCAM-1: Rac1 activation (gone by 12')	N/D
Van Buul et al., J Cell Biol 2007. [97]	Anti-ICAM-1 coated polystyrene beads	HUVEC	YES 10' XL ICAM-1: RhoA and Rac1 activation (gone by 30')	N/D
Martinelli et al., Mol Biol Cell 2009. [103]	"Ligate" with anti-ICAM-1 primary alone or crosslink with secondary	Immortalized rat brain MV EC	YES 2'-45' ligation with anti-ICAM1 ab alone: RhoA activation 2-30' XL ICAM-1: RhoA activation	N/D

Table 1. Summary of publications which observe RhoA and/or Rac1 activation as well as cytoskeletal changes in endothelial cells after ICAM-1 or VCAM-1 crosslinking (XL). ND = Not determined. Papers are presented in chronological order.

Given the evidence presented in the existing literature, we set out to ask if ROS are involved in the activation of RhoA downstream of ICAM-1 or VCAM-1 signaling. To visualize ICAM-1 clustering and subsequent changes in the endothelial monolayer, I performed immunofluorescence experiments. TNF-stimulated HUVECs were treated with anti-ICAM-1 antibody alone for 30 minutes, followed by fixation in 3.7% formaldehyde solution or treated with anti-ICAM-1 antibody for 30 minutes, washed with serum-free endothelial media, and treated with Alexafluor-488 conjugated goat anti-mouse secondary antibody for 60 minutes prior to fixation. Cells that were treated with primary antibody alone were stained with 488-conjugated goat anti-mouse antibody post-fixation. All cells were stained with Texas-red conjugated phalloidin to label actin. As seen in figure 13A, addition of the crosslinking secondary antibody results in significant ICAM-1 clustering, stress fiber formation, and large gaps in the endothelial monolayer (asterisk).

As described above, RhoA and Rac1 activation has been observed in endothelial cells after crosslinking surface cell adhesion molecules (ICAM-1 or VCAM-1) or after monocyte adhesion. To corroborate previously published findings, I performed RBD and PBD pulldowns (as described in chapter 2) after ICAM-1 crosslinking. ICAM-1 was crosslinked on TNF-stimulated HUVECs for 15 minutes using soluble antibodies, and RhoA and Rac1 activity assays were performed. As shown in figure 13B, both RhoA and Rac1 are activated in response to ICAM-1 crosslinking.

Rac1 activation by ICAM-1 has been shown previously by Van Buul et. al although the focus of this work was on ICAM-1 activation of RhoG and subsequent apical cup assembly [97]. Rac1 activation downstream of RhoG has been described to occur through a complex with DOCK180 and ELMO [171]. To ask if RhoG is required for Rac1 activation downstream of ICAM-1, I used siRNA to knockdown RhoG in HUVECs followed by ICAM-1 crosslinking. Figure 13C shows that Rac1 is still activated, although not as robustly, in HUVECs in which RhoG has been knocked down. This indicates that a pathway independent of RhoG/DOCK180/ELMO may be involved.

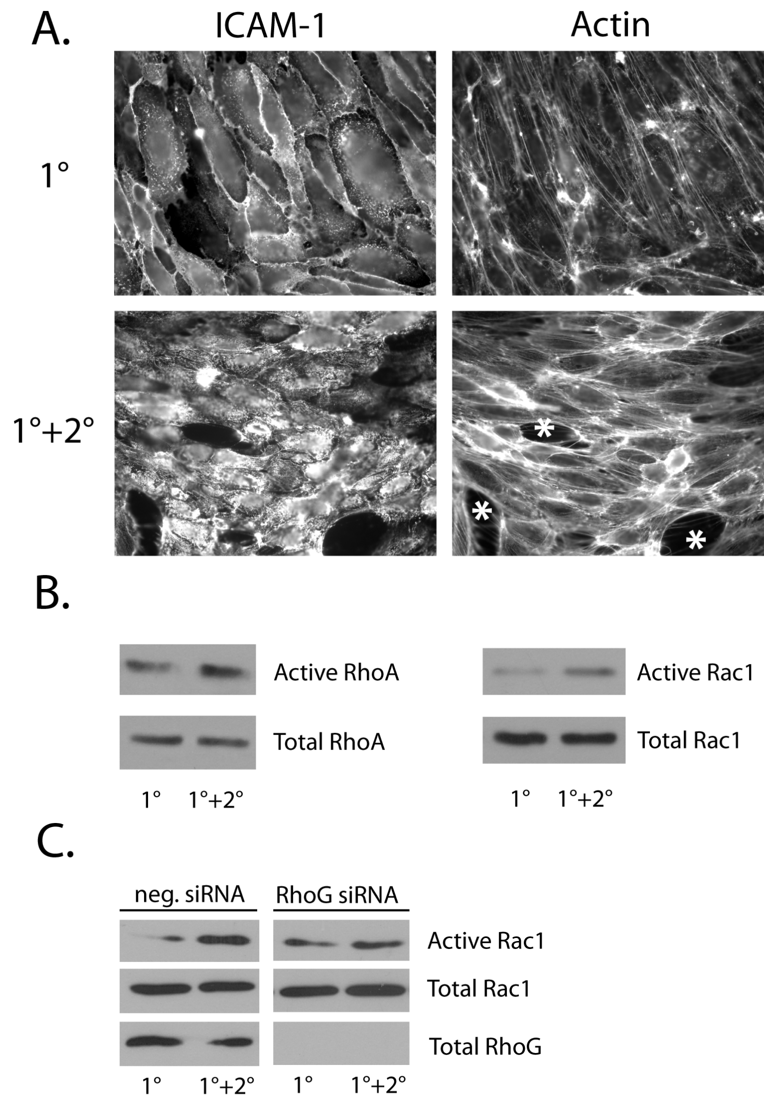


Figure 13. ICAM-1 crosslinking results in stress fiber formation and activation of RhoA and Rac1. A. HUVECs were grown to confluence on matrigel coated glass coverslips and treated with 5ng/mL TNF-alpha overnight. Cells were treated with anti-ICAM antibody for 30 minutes and fixed in 3.7% formaldehyde solution (1° only), or crosslinked with Alexafluor-488 conjugated goat anti-mouse secondary for 60 minutes (1°+2°) prior to fixation. Cells that received primary antibody only were stained with Alexafluor-488 conjugated goat-anti mouse secondary post-fixation. All cells were stained with Texas red conjugated phalloidin. Crosslinking ICAM-1 results in stress fiber formation and gaps in the monolayer (asterisks). B. HUVECs were grown to confluence and treated with 5ng/ml TNF-alpha overnight. Cells were treated with anti-ICAM primary alone or ICAM-1 was crosslinked with goat-anti mouse secondary for 15 minutes. RBD and PBD assays were performed as described in chapter 2. Crosslinking ICAM-1 stimulates RhoA and Rac1 activation. C. RhoG was knocked down in HUVECs using Oligofectamine. 48 hours post transfection, HUVECs were treated with TNF overnight and ICAM-1 was crosslinked as described above.

ICAM-1 crosslinking does not activate C20S RhoA

Given our work on regulation of RhoA by ROS (presented in chapter 2), I wanted to ask if ICAM-1 activation of RhoA involved ROS. To do so, I used C20S RhoA-myc as a ROS-insensitive RhoA mutant. To show that this mutant of RhoA is not activated by ROS, HUVECs were infected with adenovirus to express either wildtype or C20S RhoA-myc, and treated with antimycin A to stimulate ROS production (see figure 4, Chapter 2). RhoA activity assays show that wildtype RhoA is significantly activated by antimycin A, whereas C20S RhoA is not (figure 14A). As shown in figure 14B, ICAM-1 crosslinking activates wildtype but not C20S RhoA, indicating a role for ROS in RhoA activation downstream of ICAM-1. However, as a control, I confirmed that both wildtype and C20S RhoA can

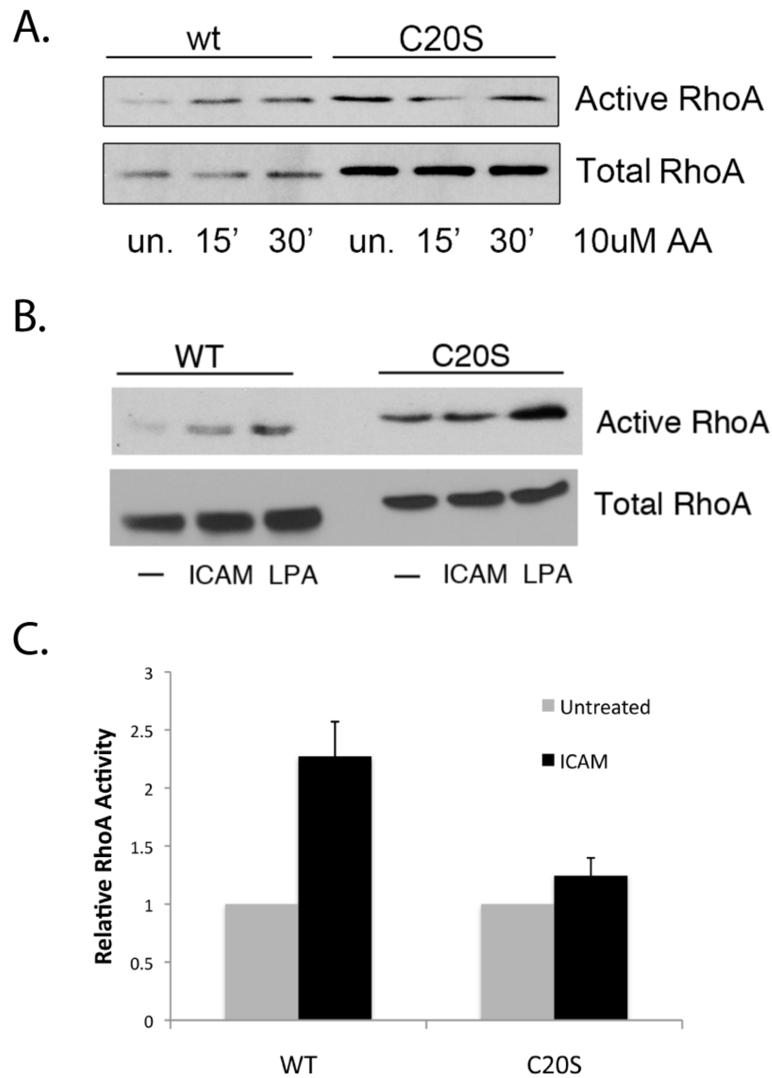


Figure 14. C20S RhoA is resistant to ROS and is not activated by ICAM-1. A. HUVECs expressing wildtype (wt) or C20S RhoA-myc were treated with antimycin A (AA) for 30 minutes, and RBD pulldown assays performed as described. AA does not activate C20S RhoA-myc. B. HUVECs expressing either wt or C20S RhoA-myc were stimulated with TNF, and ICAM-1 was crosslinked for 15 minutes using anti-ICAM coated beads. LPA treatment for 3 minutes serves as a positive control. Wildtype RhoA is activated by ICAM-1 crosslinking and LPA, whereas C20S RhoA is not. C. Quantitation of RhoA activity assays after ICAM-1 crosslinking, representative of 2 independent experiments.

Chapter 4: Clinical Significance and Future Directions

Clinical Significance

In this chapter, I will begin by discussing the clinical relevance of the work presented in this thesis. Two scenarios involving ROS and inflammation will be discussed: acute inflammation, during ischemia-reperfusion injury (IRI); and chronic inflammation, in the context of atherosclerosis. This discussion of clinical relevance will lead into topics and questions for future study.

Ischemia-reperfusion injury (IRI)

IRI is an acute inflammatory event which occurs when circulation is restored to ischemic tissues, for example, in the context of thrombosis, hemorrhagic shock, or organ transplantation. Although it is counter intuitive to consider that restoration of blood flow to an ischemic tissue could cause more damage, IRI has in fact been known as a serious pathological consequence of therapeutic intervention for quite some time. Hearse et al. were the first to show that reoxygenation of hypoxic rat heart resulted in further damage to the tissue rather than improvement [172]. Over the years, a significant effort has been made to understand the pathophysiology of IRI. These mechanisms include cellular calcium loading, acute inflammatory response, and perhaps most importantly the production of ROS and RNS [157].

Although endothelial cells have numerous mechanisms to eliminate ROS and RNS, during IRI these defenses are overwhelmed. This leads to increased

vascular permeability, vessel leakage, and an acute inflammatory response which exacerbates and promotes further damage. The importance of ROS to the pathophysiology of IRI is demonstrated by *in vivo* studies in which ROS/RNS during IRI was eliminated by administration of a radical scavenger. For example, during lung transplant in the rat [173] or warm ischemia in the mouse [166], administration of N-acetyl cysteine significantly attenuated vascular injury and inflammation. We found similar results, using a lung hilum clamp model in the mouse (see Figure X). Similar findings were obtained using NADPH oxidase (p47phox-/-) mice or mice treated with the NADPH oxidase inhibitor apocynin [174].

As discussed in Chapter 1, Rho GTPases have been shown to be critical players in the regulation of vascular permeability. Furthermore, inhibition of Rho kinase (a primary effector of RhoA) has been shown to attenuate some of the pathological consequences of IRI. For example, in a rat model of cardiac IRI, pretreatment with Y27632 prevented the cytoskeletal changes of endothelial cells associated with IRI [175]. A mouse model of cardiac IRI showed that inhibition of Rho kinase significantly reduced infarction after IRI [176]. Studies have been performed in other organs with similar results, with the most recent study showing that Y27632 reduces leukocyte infiltration in a rat model of renal IRI indicating a protective effect on vascular permeability and inflammation [177].

Given that we have shown that Rho GTPases can be activated by ROS, it is reasonable to propose that ROS may play a role in GTPase regulation and subsequent increases in vascular permeability caused by IRI. *In vitro* studies

implicate ROS as a mechanism regulating Rho GTPases and changes in vascular permeability which occur during hypoxia and reoxygenation. Its important to note that hypoxia is not equivalent to ischemia, as oxygen tension stays high over some time in some tissues (namely the lung) despite lack of blood flow. Regardless, these studies provide valuable information about the potential role of ROS and Rho GTPases during IRI. An early paper by Lum and colleagues, examined bovine pulmonary microvascular endothelial cells and their response to hypoxia and reoxygenation [178]. They found that reoxygenation induced a rapid increase in vascular permeability which was associated with increased stress fiber formation and spindle shaped cells. They also measured increased ROS production during reoxygenation, and that reducing ROS production (by pre-treatment with SOD or catalase) attenuated the increase in vascular permeability. Interestingly, they found that SOD was effective only if it was added during hypoxia. Similarly, overexpression of SOD in human aortic endothelial cells was found to prevent reoxygenation-induced actin organization after hypoxia [179]. These results indicates that perhaps hypoxia causes a depletion of antioxidant enzymes (such as SOD), which are then overwhelmed by the ROS production which occurs during reperfusion.

RhoA and Rac1 activity has also been measured using pulldown assays (as described in Chapter 2) during hypoxia and reoxygenation [59, 180]. Wojciak-Stothard et al. show that hypoxia results in decreased ROS production and that reoxygenation returns ROS levels to normoxic conditions. Furthermore, hypoxia resulted in decreased Rac1 activity and elevated RhoA activity. Experiments

using dominant negative Rac1 implicate downregulation of RhoA as downstream of elevated Rac1 activity. This corroborates with the Bar-Sagi pathway [57] described in Chapter 1. However, based on the data described in Chapter 2, we would expect that decreased ROS levels may result in lower levels of activity for both Rac1 and RhoA and that Rac1 activation may lead to RhoA activation via ROS. This is of course a simplistic assumption based on the data presented and its likely that a huge number of factors are affected when cells are exposed to hypoxia and reoxygenation. However, it does indicate that ROS levels during hypoxia/reoxygenation *in vitro* are regulators of Rho GTPases and that a similar mechanism may be occurring during IRI *in vivo*.

Atherosclerosis

Atherosclerosis is an inflammatory disease characterized by the infiltration of leukocytes and smooth muscle cells across the endothelium in response to the accumulation of pro-atherogenic factors, namely oxidized lipids. This accumulation of cells and lipids results in lesions within the vessel wall called "plaques." Originally, it was thought that plaques consisted mainly of lipids. However, recruitment of monocytes to the vessel wall and subsequent transformation into "foam cells" was shown to be an important event preceding plaque formation [181]. Analysis of the cellular composition of plaques showed that macrophages, T cells, and smooth muscle cells were a significant component of plaques [182]. The identification of these cells in plaques opened the door to a body of work investigating the mechanisms by which cells invade into the endothelium during plaque formation.

As expected, the cell adhesion molecules ICAM-1 and VCAM-1 have been shown to be an important component of atherosclerotic plaque formation. Selectins have also been shown to be important for plaque formation, however, they will not be discussed here.

Atherosclerosis is known to occur preferentially at sites of arterial branching or curvature where there is turbulent blood flow. This is likely due to two main factors, the first being that lipids tend to accumulate at these sites. Secondly, EC expression of cell adhesion molecules such as ICAM-1 and VCAM-1 has also been shown to be upregulated in regions of turbulent flow and shear stress. This likely contributes significantly to the development of plaques in these regions. Both ICAM-1 and VCAM-1 have been shown to be expressed on ECs in atherosclerotic plaques *in vivo* [183, 184]. *In vitro* experiments show that the accumulation of oxidized lipid enhances the expression of cell adhesion molecules in activated ECs [185]. Importantly, in rabbits fed an atherogenic diet, the expression of VCAM-1 occurs very early, much before the first appearance of neointimal macrophages [186, 187]. This indicates that ICAM-1 and VCAM-1 are likely upstream of the inflammatory events which mediate atherogenesis, rather than their elevated expression being a consequence of monocyte recruitment.

A number of studies using knockout animals have shown the importance of cell adhesion molecules to plaque formation. ICAM-null animals on either a standard C57Bl/6 background or ApoE-/- background have been shown to have reduced plaque formation [188, 189]. Results seem to highly depend on the timepoint at which the animals are studied and the diet the animals are fed.

Bourdillon et al. found reduced plaque formation in ICAM-null animals at all timepoints studied (6-20 weeks) if the animals were fed a standard “chow” diet [190]. However, the absence of ICAM was not protective at early (6 weeks) or late timepoints (20 weeks) if the animals were fed a high-fat atherogenic diet. VCAM-1 null animals have also been studied and found to have a significant reduction in plaque formation [191]. Interestingly, Cybulsky and colleagues did not find that ICAM deficiency was atheroprotective.

The role of ROS in atherogenesis has focused largely on the contribution of oxidized lipids to plaque formation. Leukocytes at the site of plaque formation generate ROS which further oxidizes LDL into highly oxidized LDL, which in turn is taken up by macrophages to form foam cells. These foam cells become embedded into the vessel wall and promote further plaque formation by secretion of growth factors, which attracts more leukocytes and smooth muscles to perpetuate this process. *In vitro* experiments have shown that expression of NADPH oxidase is upregulated under conditions of turbulence or oscillatory shear stress, which is pro-atherogenenic [192]. The opposite is true under conditions of laminar shear stress which is atheroprotective. *In vivo*, deletion of NADPH oxidase (p47phox -/-) has been shown to protect mice from development of plaques when in a hypercholesterolemic background (apoE -/-) [193]. These mice also had significantly reduced production of aortic ROS production. A study of atherosclerotic human coronary arteries showed that ROS production was enhanced and that oxidized LDL is spatially associated with a subunit of the NADPH oxidase complex (p22phox) [194]. Furthermore, a number of vasoactive

factors which are known to generate ROS/RNS such as angiotensin II, thrombin, and TNF have been found in human atherosclerotic plaques.

It is tempting to speculate that ROS production during the development of atherosclerotic plaques could involved regulation of Rho GTPases. As described throughout this thesis, Rho GTPases regulate vascular permeability and leukocyte transendothelial migration through their actions on the cytoskeleton. These processes are both highly involved in the pathogenesis of atherosclerosis. In addition to their role as modulators of the cytoskeleton and endothelial barrier function, Rho GTPases may have a role in activation of endothelial cells during the early stages of atherosclerosis.

Hypoxia and reoxygenation and the resulting production of ROS has been shown to stimulate expression of VCAM-1 [195]. Importantly, this is abolished in cells expressing dominant negative Rac1, indicating Rac1 is upstream of VCAM-1 expression. Rac1 is also implicated in the enhanced expression of cell adhesion molecules when cells are stimulated with TNF alpha [196] or when cells are subjected to shear stress [197]. Both of these situations involve elevated ROS production and Rac1 activation. The common link between all of these studies is likely to be Rac1's important role as an upstream activator of NF-kB, which is known to promote the activation of a multitude of genes involved in the inflammatory response including ICAM-1 and VCAM-1 [198, 199]. RhoA has also been associated with increased expression of cell adhesion molecules in studies using thrombin, a well studied activator of RhoA/Rho kinase and NF-kB [200].

Future Directions

Understanding the mechanisms which regulate leukocyte transmigration is critical to developing therapies for the large number of pathological situations which involve inflammation. In this thesis, I have presented preliminary evidence for a potential pathway involving regulation of Rho GTPases by ROS during leukocyte TEM. This hypothesis is based on findings presented in Chapter 2, in which we showed that ROS can directly regulate the GTPase RhoA and that this mechanism of regulation requires the presence of two cysteine residues in RhoA. In the preliminary work presented in Chapter 3, I have shown that ICAM-1 activation stimulates RhoA and Rac1, and that C20S myc-RhoA is resistant to ICAM-1 activation. This indicates that ROS may potentially be involved in RhoA activation after ICAM-1 engagement.

In future work, it will be critical to determine the role of ROS in mediating TEM and whether regulation of RhoA by ROS has a role in TEM. In figure 12, I have shown that inhibiting ROS production in endothelial cells inhibits the transmigration of monocytes. This result is preliminary, but is in agreement with the findings of others who have shown that inhibiting either ROS or RNS in endothelial cells results in decreased leukocyte TEM [103, 201]. These experiments need to be repeated. Inhibitor studies have caveats due to the non-specific nature of many inhibitors used to inactivate ROS/RNS producing enzymes. However, these studies, if carefully controlled, can provide useful information on the sources of ROS/RNS involved in TEM. It would also be useful to measure ROS/RNS production in endothelial cells upon leukocyte adhesion. I

have attempted these experiments, however, I have had great difficulty due to the sensitive nature of the dyes used. A large number of oxidation sensitive dyes are available to test, which greatly increases the likelihood of successfully measuring ROS production in ECs after leukocyte adhesion.

In addition to inhibitor studies and measurement of ROS production, a critical experiment is to measure leukocyte TEM across endothelial monolayers which express either wildtype or ROS-resistant C20S myc-RhoA. If our hypothesis is correct, expression of C20S RhoA will reduce leukocyte TEM. These experiments would have to be carefully controlled to show that expression of C20S RhoA does not affect leukocyte adhesion or ICAM-1 clustering, as RhoA has been shown to be involved in the expression and function of cell adhesion molecules [202].

In addition to TEM experiments utilizing endothelial cells expressing wildtype or C20S RhoA, it will important to do GTPase activation assays after leukocyte adhesion. These experiments can be difficult given the potent proteases present in many leukocytes. Using high concentrations of protease inhibitors during lysis and increasing the amount of GST proteins used for pulldown assays (thereby decreasing the incubation time) should reduce proteolysis of proteins. Experiments utilizing leukocyte adhesion are also valuable in that they provide insight into whether ICAM-1, VCAM-1, or both cell adhesion molecules are involved in RhoA activation and potentially ROS activation of RhoA. This can be achieved in experiments comparing the response of endothelial cells to the adhesion of granulocytic leukocytes (which primarily

use ICAM-1) versus monocytic leukocytes (which use both ICAM-1 and VCAM-1). Similar experiments can be performed using blocking antibodies to block either ICAM-1 or VCAM-1. VCAM-1 has been shown to be present in “cups” which surround transmigrating leukocytes [101] and that Rac1 activation downstream of VCAM-1 results in ROS production which is required for TEM [201]. It will interesting to repeat crosslinking experiments using anti-VCAM-1 antibody, and measure Rac1 and RhoA activation.

In addition to identifying a role for ROS, and regulation of RhoA by ROS during TEM, it will be important to investigate the mechanisms of ROS production in ECs after leukocyte adhesion via ICAM-1 or VCAM-1. A critical experiment is the use of ICAM-1 or VCAM-1 truncated mutants in which the cytoplasmic tail has been deleted. Experiments using truncated mutants of ICAM-1 have shown that the cytoplasmic tail is important for RhoA activation [203]; ICAM-1 localization and cup assembly [204]; and leukocyte transmigration [85, 205]. Using adenovirus to express either GFP-tagged full length or cytoplasmic tail deleted ICAM-1 would allow us to ask if ICAM-1’s cytoplasmic tail is required for ROS production, Rac1 and RhoA activation, stress fiber formation, and increased permeability.

As described above, VCAM-1 activation has been shown to result in Rac1 activation, NADPH oxidase activation and ROS production. In preliminary data, I have shown that ICAM-1 can activate Rac1 (figure 13). ICAM-1 engagement has been shown to result in ROS production through xanthine oxidase [206] and NOS production through iNOS [103]. Given the existing literature on VCAM-1, it

is plausible to consider the hypothesis that ICAM-1 activation may also result in ROS production through Rac1 NADPH oxidase. Given our laboratory's expertise in identification of GEFs, it would be feasible to also identify GEFs downstream of ICAM-1 activation leading to Rac1 activation and NADPH oxidase activation. It may be the case that both ICAM-1 and VCAM-1 can activate Rac1 but only VCAM-1 results in NADPH oxidase activation. This would be a perfect example of the critical importance of localization to ROS signaling. Although both cell adhesion molecules can activate Rac1, only signals derived from one signaling cascade, presumably mediated through unique binding partners and scaffolding proteins, result in NADPH oxidase activation. This has been shown for S1P and VEGF. Both growth factors potently activate Rac1, however, only VEGF results in ROS production via NADPH oxidase in endothelial cells [49]. Should this be the case for ICAM-1 and VCAM-1, investigating the unique molecular players involved would be an interesting and important direction for future research.

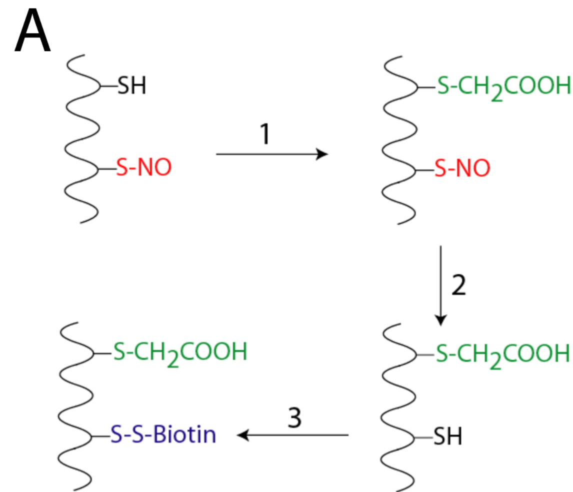
Appendix

In this appendix, I describe experiments and approaches which I have attempted. I present a brief summary of the approach, potential issues with these experiments and possibilities for future study.

Mass spectrometry/proteomics approaches

The identification of cysteine oxidative modification in Rho GTPases is a critical component of this project. In describing the mechanism for redox-mediated Rho GTPase regulation, Sharon Campbell's group obtained and published mass spectrometry results showing chemical intermediates and oxidative modification (disulfide bond formation) [63, 64]. I attempted to identify cysteine modifications of RhoA, immunoprecipitated from cells using two methods. The first method, is a brute force mass spectrometry approach in which RhoA was expressed in Hela cells or fibroblasts as a myc-tagged protein, and immunoprecipitated after treatment of cells with media alone or media containing hydrogen peroxide. A high, non-physiological level of peroxide was used (1-5mM) to attempt to form an irreversible oxidative modification. Due to a variety of logistical and technical issues, I was not able to obtain reproducible data with this approach. The second approach is a biochemical approach in which oxidized cysteines in proteins are biotinylated. This technique has a number of variations [207-209]. Common to all techniques is the general approach involving three steps: (1) blocking of free thiols (cysteines) (2) reduction of oxidized cysteines (3) labeling of newly formed cysteines. A diagram of this general procedure is shown in Figure 15A, using S-nitrosylation as an example. In Figure 15B, I present a

preliminary experiment in which cells were treated with an NO donor, SNAP, followed by the oxidized-cysteine labeling protocol. In this preliminary experiment, RhoA appears to be nitrosylated although the specific residue was not identified. An appropriate next step would be repeating this procedure with cysteine to alanine RhoA mutants. In collaboration with Aaron Hobbs from the Campbell lab and Rob Dekroon from the Systems proteomics facility (Oscar Alzate), we have attempted to identify oxidative modifications using purified recombinant RhoA GTPase. Our approach essentially follows the procedure outlined above, with the exception that blocking of free thiols in step 1 and labeling of newly formed thiols after reduction (step 3) both utilize fluorescent-tagged probes. This allows independent measurement of “free cystienes” and “oxidized cysteines” providing a quantitative measure of the extent of cysteine oxidation. These studies to date have proved to be difficult, with a variety of factors causing protein degradation or denaturation and unintended oxidation. This remains a work-in-progress.



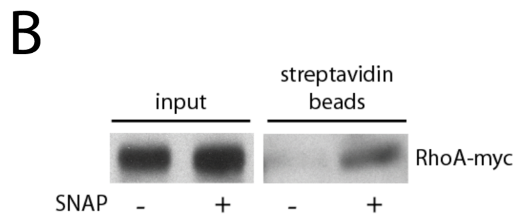


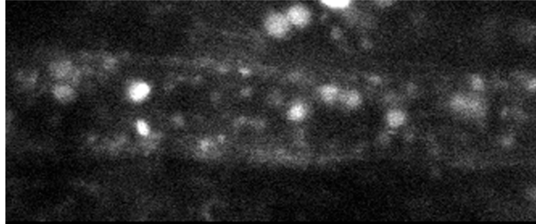
Figure 15. Labeling oxidizing cysteines. A. General approach to labeling oxidized cysteines beginning with (1) blocking of free cysteines with iodoacetic acid or other irreversible thiol reacting compound (2) reduction of oxidized cysteines (3) labeling of newly formed free cysteines with biotin or fluorophore-tagged thiol reacting compound. B. Hela cells expressing RhoA-myc were subjected to the cysteine labeling procedure using iodoacetic to block free cysteines and DTT to reduce oxidized cysteines, followed by labeling with biotin. Streptavidin beads were used to isolate biotinylated proteins and the isolated sample was blotted with anti-myc antibody. Treatment of cells with SNAP (an NO donor) resulted in detection of cysteine oxidation of RhoA-myc.

Intravital microscopy

Visualization of leukocyte-endothelial interactions is critical to our understanding of the basic mechanisms of leukocyte transmigration and inflammation. Advances in mouse models and microscopy make intravital microscopy more accessible now than ever before. Many of the intravital studies in this field have used a cremaster model. Although this model is well characterized and used frequently, it requires significant technical and surgical skill as well as specialized preparation of the microscope stage. In preliminary studies, I have utilized a mouse dermal model of inflammation using the mouse ear. This approach is much more straightforward than the cremaster model, and can be performed on any confocal microscope with a heated stage. One disadvantage of this model is the inability to directly inject cytokine (for example TNF-alpha) into the tissue, as the mouse ear is simply too thin. As an alternative, acute dermal inflammation can be stimulated with a topical irritant, for example croton oil. There are multiple methods available for visualizing leukocytes. A transgenic mouse can be used which expresses GFP on specific leukocyte types. For example, the Cx3cr1 mouse from Jackson labs expresses EGFP on monocytes, dendritic cells, and natural killer cells. One can also directly inject fluorophore-conjugated antibodies against leukocyte markers. This would allow tagging of specific leukocyte types (for example monocytes vs neutrophils), but has the disadvantage that it is quite expensive given the expense of antibodies and the relatively large quantities of antibody used per experiment. The last approach is the simplest, and involves injection of a dye which labels all intravascular cells with mitochrondria. This dye,

Rhodamine 6G, is readily available and inexpensive. Although this approach is straightforward, it does have the disadvantage that it is non-specific for any leukocyte in the bloodstream. In addition to labeling leukocytes, it is advantageous to label the vascular space. Injection of fluorescently labeled dextran is commonly used, although transgenic animals or fluorophore-conjugated antibodies can also be used. In figure 16, I present two images from an intravital imaging experiment demonstrating the ability to visualize the vascular space and leukocytes *in vivo* using a mouse ear model. All of these experiments are performed under an authorized IACUC protocol #10-210.0 .

A



B

Figure 16. Examples of intravital microscopy using a mouse ear model. A. Leukocytes were labeled in vivo with Rhodamine 6G via tail vein injection and visualized in the mouse ear using a 25X water objective. B. The vascular space is visualized using Rhodamine-dextran injected via tail vein.

Experiments with leukocytes

An important part of this project is to translate experimental results from antibody crosslinking experiments to physiologically relevant experiments using leukocytes as the stimulus for endothelial signaling events. However, this approach poses a multitude of complications for biochemistry experiments. The first, is that leukocytes interact with endothelial cells with a variety of different cell adhesion molecules. This results in an array of different signaling events, and significantly complicates analysis of results from biochemistry experiments. Not all leukocytes and leukocyte cell lines adhere to endothelial cells via the same adhesion molecules, therefore, this can be used to delineate which adhesion molecules are involved with a given signaling pathway. The second problem with using leukocytes is that they express potent proteases. This was a significant source of frustration for this project, as I found that leukocyte proteases caused significant degradation of endothelial proteins. Figure 16 shows total levels of VE-cadherin and PECAM (both endothelial proteins) when lysed in the presence of increasing amounts of THP-1 cells in SDS containing sample buffer. Its important to note that this effect was not dependent on adhesion, and occurred when leukocytes were simply mixed with endothelial lysates. This can be dealt with by lightly fixing leukocytes [210]. This approach is commonly used but does pose issues with using a physiologically irrelevant stimulus. As shown in Figure 16, lightly fixing leukocytes prior to combining them with endothelial cells prevents endothelial protein degradation. The second approach is to adjust experimental methods such that they can be performed more quickly, and also

pre-treatment of leukocytes with high doses of protease inhibitors prior to experiments. I have not tried this approach.

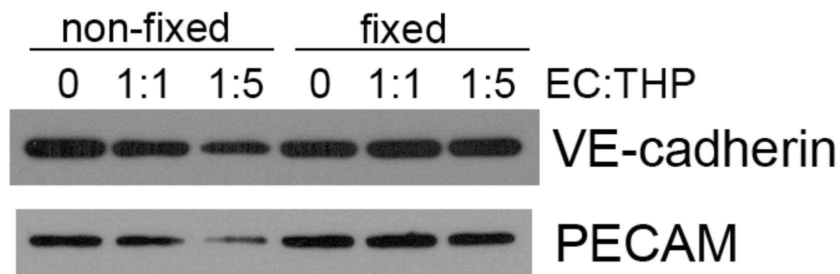


Figure 17. THP cells result in degradation of EC proteins. EC's were mixed with THP cells in either a 1:1 or 1:5 ratio and lysed in sample buffer containing 2% SDS, 50mM DTT, and 5% BME. THP cells were left non-fixed or fixed gently with 2% paraformaldehyde and washed extensively. The total cell lysates were blotted for endothelial specific markers, VE-cadherin and PECAM. THP cells cause a significant reduction in VE-cadherin and PECAM levels when not fixed, however, this effect is abrogated when THP cells are fixed indicating a likely role for leukocyte proteases.

Rac1/SOD1 interaction

Feedback loops are hypothesized to be important components of redox-mediated signaling pathways, as they are in many other signaling pathways. These pathways can work in multiple ways, for example by coupling ROS/RNS producing enzymes with ROS/RNS scavenging enzymes through a common factor. A recent study demonstrates the interaction of Rac1 and superoxide dismutase (SOD1), and proposes a model in which the interaction between Rac1 and SOD1 serves as a “redox sensor” [159]. They propose that under reducing conditions, Rac1-GTP and SOD1 interact and that SOD1 stabilizes the active GTP-bound Rac1. This drives ROS production through NADPH oxidase. The localized accumulation of ROS, specifically hydrogen peroxide through spontaneous or SOD1-driven dismutation, leads to dissociation of SOD1 from Rac1. This dissociation promotes GTP hydrolysis to GDP, Rac1 inactivation, and presumably shuts down NADPH oxidase activity.

To explore the interaction between Rac1 and SOD1 we have generated several tools. We have generated GST-tagged SOD1 to use in pulldown assays, in which we ask if different cellular treatments promote or inhibit endogenous Rac1 association with SOD1. For example, does Rac1 activation by for example EGF stimulate Rac1’s association with SOD1. Does alteration of cellular redox state, either by exogenous or endogenous ROS or RNS, affect Rac1’s association with SOD1? We have also generated myc-tagged wildtype, C18A, and C18S Rac1. These proteins can be expressed in cells, and used to ask if the redox-state of Rac1 affects its binding to SOD1. For example, does activation of

Rac1 by ROS promote its binding to SOD1 and is this effect abolished with redox-insensitive C18A or C18S Rac1? In a preliminary experiment, I have been able to confirm that endogenous Rac1 can be pulled out of cell lysates with GST-SOD1. However, no change was observed when cells were treated with peroxide to oxidize Rac1 or when cells were treated with EGF to activate Rac1. Due to time constraints, I was not able to further pursue these experiments.

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