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Complete List of Authors:	Chichger, Havovi; Anglia Ruskin University, Biomedical and Forensic Sciences Rounds, Sharon; Providence VA Medical Center, Pulmonary, Critical Care and Sleep Medicine; Brown University, Medicine Harrington, Elizabeth; Brown University, Medicine; Providence VA Medical Center, Research Services
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FORUM REVIEW ARTICLE

**Endosomes and Autophagy: Regulators of Pulmonary Endothelial Cell
Homeostasis in Health and Disease**

Havovi Chichger¹, Sharon Rounds^{2,3}, Elizabeth O. Harrington^{2,3}

¹Biomedical Research Group, Department of Biomedical and Forensic Sciences, Anglia
Ruskin University, Cambridge, United Kingdom

²Vascular Research Laboratory, Providence Veterans Affairs Medical Center,
Providence, RI, USA

³Department of Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine,
Alpert Medical School of Brown University, Providence, RI, USA

Correspondence and request for reprints:

Elizabeth O. Harrington, PhD

Research Services, 151

Providence VA Medical Center

830 Chalkstone Avenue

Providence, RI 02908

Phone: 401-273-7100

Email: elizabeth_harrington@brown.edu

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ABSTRACT

Significance: Alterations in oxidant/antioxidant balance injure pulmonary endothelial cells and are important in the pathogenesis of lung diseases, such as Acute Respiratory Distress Syndrome (ARDS), ischemia/reperfusion injury, pulmonary arterial hypertension (PAH), and emphysema.

Critical Issues: While the role of autophagy in cellular homeostasis in pulmonary disease has been investigated, the role of the endosome in the lung vasculature is less known. Furthermore, autophagy can either decrease or exacerbate endothelial injury, depending upon inciting insult and disease process.

Recent Advances: The endosomal and autophagic pathways regulate cell homeostasis. Both pathways support recycling or degradation of macromolecules or organelles, targeted to endosomes or lysosomes, respectively. Thus, both processes promote cell survival. However, with environmental stress or injury, imbalance in endosomal and autophagic pathways may enhance macromolecular or organelle degradation, diminish biosynthetic processes, and cause cell death.

Future Directions: Diseases affecting the pulmonary endothelium, such as emphysema, ARDS, and PAH, are linked to altered endosomal or autophagic processing, leading to enhanced degradation of macromolecules and potential cell death. Efforts to target this imbalance have yielded limited success as treatments for lung injuries, which may be due to the complexity of both processes. It is possible that endosomal trafficking proteins,

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such as Rab GTPases and LAMTORs, may be novel therapeutic targets. While endocytosis or autophagy have been linked to improved function of the pulmonary endothelium *in vitro* and *in vivo*, further studies are needed to identify targets for modulating cellular homeostasis in the lung.

Endosome Transport and Autophagy

The endosomal and autophagic pathways regulate movement of macromolecules within the cell with the primary goal of maintaining cellular homeostasis. Cells are constantly responding to intracellular and extracellular stresses and signals. In settings of increased stress or signaling, the balance of the processes may become tilted resulting in enhanced macromolecular degradation, diminished biosynthetic processes, and possible cell death. However, the overall purpose of both is to promote cell homeostasis.

Endocytosis is the active process of moving macromolecules and particles from the environment or external membrane into the cell via invagination of the plasma membrane through one of four methods: i) clatherin mediated endocytosis (CME), ii) clatherin independent endocytosis (CIE), iii) macropinocytosis, or iv) phagocytosis (80, 98). CME is dependent upon dynamin for membrane scission, whereas CIE can be dynamin dependent (caveolea or Rho-mediated) or independent (cdc42, Arf6, or flotillin) (80, 98). CME is a selective mechanism to internalize surface proteins, while CIE is thought to be more of a bulk method of endocytosis. Macropinocytosis and phagocytosis are endocytic processes driven by an actin ring formed beneath the plasma membrane engulfing either fluid droplets or solid structures, respectively. While macropinocytosis nonselectively takes up fluid, phagocytosis is initiated by the interaction of particles ($>0.5\mu\text{m}$) to be engulfed with cell receptors, including Fc γ , C-type lectin, integrins, and scavenger receptors (22). Both processes utilize small GTPases, such as cdc42, Rac, and Rabs, to mediate the actin polymerization and endocytosis, respectively (19). Once endocytosed, the vesicular contents are sorted and can be recycled back to the cell surface, enter into

the endoplasmic reticulum (ER) and Golgi complex for posttranslational modification of the cargo proteins, or targeted for lysosomal degradation, autophagy, or secreted into the cytoplasm (68) (Figure 1).

Protein families such as Rab-GTPases, ADP-ribosylation factor (Arf) G-proteins, endosomal sorting complexes required for transport (ESCRTs), and soluble N-ethylamide-sensitive factor attachment protein receptors (SNAREs) regulate the spatial and temporal control of this dynamic endosomal trafficking system. These families regulate trafficking processes through a variety of highly specialized mechanisms. Similar to the Ras GTPase superfamily, Rab GTPases require guanine exchange factors (GEFs) to promote the GDP to GTP exchange, allowing the change in conformation needed to bind to the endosomal membrane and mediate trafficking along the microtubule network (114). The majority of the 70 plus Rab GTPases are involved in endocytic processes. Rab5, and effector protein EEA1, associate with clathrin-coated endosomes, internalized at the plasma membrane, to mediate trafficking to the early endosome compartment (108). Rab4 and Rab11, in turn, mediate the short-loop and long-loop recycling of endosomes back to the plasma membrane, respectively (97). Rab7 and Rab9, however, promote the formation of late endosomes of cargo sorted in the trans-Golgi network (41). Early endosomes at the cell periphery undergo fusion and fission with other endosomes to create larger endosomes within the cell to allow sorting of the macromolecular contents during trafficking.

Arf G proteins also interact with GEFs and guanine activating proteins (GAPs) to regulate GDP/ GTP exchange and GTP hydrolysis, respectively. Arf proteins localize throughout the cell at membrane surfaces, including the plasma, endosomal, lysosomal, and secretory membranes (17). Six Arf proteins have been identified whose functions include recruitment of proteins which coat the membrane during endocytosis to promote sorting, enzymes which modify membrane lipid content, and proteins involved in cytoskeletal tethering, scaffolding and folding (17). Unlike Ras, Rho, and Rab GTPases, Arf G-proteins are myristoylated, which promotes their localization to membranes. Arf1 and Arf3-5 are primarily in the ER-Golgi complexes regulating retrograde transportation from the Golgi to ER and at the trans-Golgi network. Arf6 is localized at the plasma membrane and is involved in cortical actin distribution, endosomal trafficking, and rapid endosomal recycling via interactions with the microtubule motor adaptor protein, JNK-interacting protein-4 (JIP4) (17). Arf1 and Arf6 regulate intercellular junctions, adherens and tight junctions, through the regulation of cadherin molecules at the cell surface (51, 111).

In contrast to Rab proteins, ESCRT proteins play a significant role in the delivery of cargo to the lysosome via the generation of multivesicular bodies (MVB) (67). The ESCRT protein complex is formed at endosomes during MVB sorting by sequential recruitment of ESCRT-0, -I and -II and the ubiquitinated cargo. The ESCRT proteins form a subdomain on the endosome, which then invaginates incorporating the ubiquitinated cargo. ESCRT-III functions to bud off the intraluminal vesicles within the MVB. Once MVB fuse with lysosomes the content of the intraluminal vesicles is degraded (9). Perturbations in any of these highly regulated pathways can result in disordered trafficking of key molecules.

ESCRT complex has been implicated as having a role in the maturation process of the autophagosome (54, 73).

SNARE are a superfamily of proteins involved in the membrane fusion of intracellular organelles, with the exception of the mitochondria (89). For membrane fusion to occur, SNARE proteins form a four-helix bundle on the adjacent membranes complex with one R-SNARE (or vesicle (v)-SNARE) on one membrane and 3 Q-SNARE (or target cell (t)-SNARE) proteins on the other membrane (89). SNARE proteins mediate merging of the membranes in close proximity and provide energy to promote the fusion of the membranes. ATP dependent SNARE folding drives this membrane fusion. Once fused, the SNARE proteins are unfolded via the ATPase N-ethylmaleimide-sensitive factor (NSF) and synaptosomal nerve-associated protein (SNAP) (89). Interestingly, the membrane fusion function of SNAREs is also crucial for exocytosis, autophagosome-lysosome fusion, and autophagosome biogenesis (20, 61).

Similar to the endocytic pathway, autophagy is both a recycling and a degradative system, whereby intracellular molecules are targeted to endosomes or lysosomes, respectively. Autophagy occurs in settings of cell stress and serves to promote cell survival. In settings of excessive autophagy, cell death ensues. As described by Mizushima and Komatsu (60), there are 3 main types of autophagy, chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy, with each resulting in the degradation of the vesicle contents (Figure 2). CMA is a precisely targeted event, whereby the protein cargo is recognized via a KFERQ-like motif, a chaperone protein, HSC70, and its co-chaperone

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3 proteins, and subsequently internalized by a lysosomal surface receptor in conjunction
4 with LAMP2A and degraded (37). During microautophagy, subcellular small molecules
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6 enter in bulk directly into late endosomes via engulfment and subsequently fuse with MVB
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8 for degradation (74). Inhibition of the ESCRT complex I blocked microautophagic
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10 degradation in MVB, suggesting a role for these proteins in this process (74) and crosstalk
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12 between the endosomal and autophagic pathways. While initially thought to be a non-
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14 selective process, there is evidence in yeast of parallel chaperone mediated events using
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16 HSC70 and KFERQ-like motif proteins in microautophagy and subsequent binding to
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18 phosphatidylserine on the lipid bilayer (94). HSC70 was also noted to serve as a
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20 chaperone in eukaryotic cells, which selectively targets cytosolic proteins to late
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22 endosomes that then undergo microautophagy (74). Additional chaperone proteins are
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24 thought to complex cargo proteins for microautophagy which targets select organelles,
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26 such as micromitophagy, microlipophagy, and micropexophagy, in yeast (94). The CMA
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28 process is upregulated in settings of oxidative stress due in part to increased LAMP2A
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30 transcription (38). Finally, macroautophagy, often just referred to as 'autophagy', is
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32 described as the most commonly occurring form of this process.
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42 Autophagy proceeds via five main steps, initiation, nucleation, elongation, lysosome
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44 fusion, and cargo degradation. Initially, an isolation membrane or phagophore is formed
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46 from the ER, Golgi, mitochondrial, plasma, or recycling endosomal membrane.
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48 Formation of the phagophore requires altered activation and/ or recruitment of a number
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50 of signaling molecules including mTOR. In settings of cellular homeostasis the mTOR
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52 complex 1 (TORC1) binds to and inactivates ULK1 (Unc-51 like autophagy activating
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kinase or autophagy-related genes-1 (Atg1)) (39). Upon cell starvation, mTORC1 is inactivated, releasing and activating ULK1, which in turn binds the membranes to initiate the phagophore formation via recruitment of other proteins, including FIP200, Atg13, and Atg101 (101). In addition to mTOR, AMP kinase (AMPK) has been shown to directly activate ULK1 via phosphorylation (39), as well as indirectly via Raptor mediated inhibition of mTOR (3, 101). With the activation of ULK1, additional proteins are recruited that are necessary for the phagophore elongation. Beclin 1, originally identified as having anti-apoptotic properties through its binding to Bcl-2, is a necessary component in autophagy via the formation of complex with class III-type phosphatidylinositol 3 kinase complex, Vps34 (29, 52). Unlike other vesicles, phagophore double membranes are formed *de novo* through the synthesis of phosphatidylinositol-3-phosphate (PI3P) and recruitment of microtubule-associated protein-1 light chain-3 (LC3) or Atg 8 and the Atg12-Atg5-Atg16 complex. Through a series of posttranslational modifications, Atg8 is covalently attached to phosphatidylethanolamine (PE) of the lipid bilayer. Atg8-PE conjugation on the outer layer of the autophagosome is thought to regulate the size of the compartment, while that on the inner surface serves in the selection of cargo that is internalized (29, 32). Once the phagophore matures into the autophagosome, the outer membrane fuses with lysosomes with the release of the inner membrane bound cargo, termed autophagic body, which is subsequently degraded and the primary cell building blocks, such as amino acids and fatty acids are recycled back to the cytosol (32).

In settings of excessive autophagy, cells may undergo autophagic or apoptotic cell death. Autophagy-dependent cell death occurs in settings of autophagic degradation of i) ferritin

(ferroptosis) (33) or ii) protein tyrosine phosphatase, non-receptor type 13 (PTPN-13; FAS-driven extrinsic apoptosis) (24), iii) via necroptosis by utilizing the necrosome scaffolding complexes in autophagy or by cellular inhibitor of apoptosis protein-1 (cIAP-1) and cIAP-2 degradation (23), and iv) by autosis, which is mediated via Na^+/K^+ ATPase (48). Apoptosis or programmed cell death is a well-studied regulated cell death (RCD) process, which may occur by signals that are initiated intrinsically or extrinsically and can occur in a caspase-independent manner. More in depth reviews on apoptosis are available (27, 85).

Regulated endosomal trafficking is key for efficient autophagy, indeed the autophagosome membrane has been demonstrated to originate from the endosomal membrane (57). Maturation of the autophagosome, by fusion with the lysosome, forms the autophagolysosome where autophagy can occur. This fusion process allows the delivery of key lysosomal components such as hydrolases to degrade autophagocytosed products, the H^+ -vacuolar ATPase to acidify the vesicle, and transporters and permeases needed to allow efficient recycling of autophagic degradation products (82). Endosomal proteins form the molecular machinery required for formation of the autophagosome. For example, several Rab GTPases (Rab11, Rab33b, Rab5 and Rab7) as well as the early endosome antigen 1 (EEA1) have all been demonstrated to impact autophagy using *in vitro* studies (8, 31, 87, 100). Several of these trafficking molecules regulate dynamics of the recycling endosome to regulate fusion between MVB and the autophagosome (Rab11), push the recycling endosome into the late endosome/ lysosome pathway (Rab7), or promote trafficking of endosomes from the Golgi to the maturing autophagosome (Rab33b) (49, 88, 90). These proteins sort endosomes early in the

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trafficking pathway, promoting the formation of the lysosome and subsequent maturation into the autophagolysosome, which enables autophagic degradation of targeted cargo. In addition to LC3/Atg5/Atg7-mediated autophagic flux, studies also indicate the presence of an independently-mediated autophagosome formation pathway (65). This LC3/Atg5/Atg7-independent autophagy is linked to the fusion of Rab9-positive endosomes with the autophagosome. Interestingly, both Rab7 and Rab9 GTPase are also involved in autophagosomal membrane formation, which occurs during mitophagy (30, 103). Recent papers suggest that Rab5 mediates the closure of the autophagosome prior to fusing with lysosomes in yeast cells (10, 115). More recently, data demonstrates that Rab5 recruits ESCRT to complete the autophagosomal scission (115). It is clear that there is cross talk between the endocytic and autophagic processes and therefore likely that Rab GTPases mediate these two key pathways, which control cellular homeostasis.

The master regulator of the lysosome, mTORC1, also plays a key role in autophagy. In nutrient-rich settings, mTORC1 reduces autophagy by inhibiting catabolic pathways, in close coordination with AMPK, and promotes biosynthesis pathways to increase cell growth (78). mTORC1 responds to several upstream stress signals, such as amino acids and growth factors, via H⁺-vacuolar ATPase and Rheb GTPase localized to the lysosome surface (77, 84). Amino acid signals from the lumen of the lysosome to the Ragulator complex result in the recruitment of mTORC1 to the lysosome (70). The Ragulator complex has therefore been the focal point of much autophagy research with studies indicating that the H⁺-vacuolar ATPase-Ragulator interaction activates the GTP-loading

of Rag GTPases, leading to mTORC1 recruitment, its activation by Rheb and the subsequent inhibition of autophagy (77). There are a range of proteins which comprise the Ragulator complex, including GTPases RagA-RagD and scaffold proteins LAMTOR1-5 (62, 77). LAMTOR1 provides the scaffold for Rag GTPases to recruit mTORC1 and the LAMTOR2/3 and LAMTOR4/5 heterodimers anchor the complex to the lysosome membrane (63). As such, inhibition of LAMTOR1 in MEF has been demonstrated to promote autophagy, through decreased mTOR phosphorylation and increased LC3II formation, in settings of nutrient deprivation (107). Interestingly, the Ragulator complex also regulates mitogen-activated protein kinase kinase (MKK) signaling from the late endosome, associated with lysosome biogenesis, membrane dynamics and organelle transport along the cytoskeleton (63). Members of the Ragulator complex, such as LAMTORs, therefore represent interesting molecular targets to modulate autophagy in settings of disease.

Function of endocytosis and autophagy in healthy endothelium

The flow of macromolecules in the endothelial cells through endosomal and autophagic trafficking is important in regulating functional vasculature, angiogenesis, and responding to aging and changes in environment. Inhibition of the basal levels of either process leads to endothelial cell apoptosis, permeability, migration and vessel regression. Conversely, excessive activation of endocytosis or autophagy can exacerbate vascular injury leading to pathological progression of diseases (Figure 3).

Endocytosis is important in the trafficking of many cell surface molecules including adherens and tight junction proteins, receptor tyrosine kinases, as well as proteins localized near the plasma membrane, such as Rho and Rac GTPases. We have shown upregulation of recycling endosomal proteins, p18 or Rab4, to increase VE-cadherin surface expression in pulmonary endothelial cells, protect against barrier dysfunction (11, 13) and promote migration, proliferation and angiogenesis (11, 12, 93). The long-loop recycling Rab GTPase, Rab11a, provides the platform for LC3-positive autophagosomes to mature and has also been shown to protect the pulmonary endothelium against leak, through improved adherens junction formation (104). Other studies have similarly shown tight junction proteins, including zonula occludens (ZO), junctional adhesion molecule-A (JAM-A), claudins and occludin to be regulated by endosomal trafficking affecting endothelial barrier function (reviewed in (86)). Receptor tyrosine kinases (RTK) were thought to function at the plasma membrane to regulate endothelial cell function, however, it is now recognized that several RTK signal instead from the cytosol via endosomes. For example, vascular endothelial growth factor receptor 2 (VEGFR2) and fibroblast growth factor receptor 1 (FGFR1) are localized to endosomes. Once bound by VEGF-A or -C, VEGFR2 internalization occurs via clathrin/ dynamin-mediated pathway (7) which then leads to Rab5 mediated trafficking to early endosomes and recycling via Rab11 (5) or via macropinocytosis (6). In addition, unbound VEGFR2 is noted to undergo constitutive endocytosis and recycling to the plasma membrane via Rab4 (40), as well as shear stress induced caveolin-dependent endocytosis of VEGFR2. Interendothelial cell junctions are transiently disrupted by some edemagenic agents, such as thrombin, via actions of Rho A and Rac 1. In settings of low levels of Rho B or deficiency, Rac 1 is

recycled via endosomes to the plasma membrane promoting barrier function. It has been shown that endosomal Rho B diminishes Rac 1 plasma localization by targeting it to late endosomes for degradation causing delayed barrier recovery (56). As Rac 1 is involved in other endothelial cell functions, such as migration, it is likely that this molecule is also impacted by endosomal trafficking.

Autophagy has been shown to regulate the levels of nitric oxide in settings of increased shear stress on static endothelial cell monolayers and *ex vivo* perfusion of the carotid artery (25) and in aging (44). The changes in shear stress correlated with increased levels of p62, beclin and LC3, autophagy markers, as well as increased eNOS and reduction in ET-1 (25) and increased Rab4 expression (106). Beclin and p62 were also reduced in the brachial artery endothelial cells of older relative to younger human subjects and mice, with corresponding decrease in endothelial dependent dilation and NO bioavailability with increase oxidative stress and inflammation (44). Further, in settings of high shear stress, autophagy was required to promote endothelial cell alignment with the flow, protect against endothelial cell apoptosis or senescence, and plaque formation (99). Similarly, autophagy attenuates oxidant injury to endothelial cells. Ha and colleagues showed that curcumin induced autophagy in human umbilical vein endothelial cells exposed to hydrogen peroxide via increased LC3II and number of autophagosomes to promote cell viability (26). Following cigarette smoke exposure, elevated autophagy and senescence have been observed in endothelial cells and lungs respectively (Figure 4). Other studies have shown induction of autophagy prevented against stroke by diminishing ROS and improving vascular relaxation of the carotid (43), improved NO

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3 signaling in endothelial cells isolated from patients with type 2 diabetes (21), and
4 improved endothelial dependent dilation in aged animals (44). Rab26, a pro-autophagic
5 GTPase which targets activated Src to LC3-positive autophagosomes, protects the
6 pulmonary endothelial barrier by stabilizing adherens junctions (18, 46). Rather
7 confusingly, Rab5a and Rab9, implicated as pro-autophagic GTPases, negatively
8 regulate barrier function in the vasculature with overexpression of the dominant-negative,
9 GDP-locked Rab9 mutant in the pulmonary endothelium attenuating bacteria- and
10 endotoxin-induced barrier disruption *in vitro* and *in vivo* (11, 105). These and other
11 studies suggest that autophagy is necessary for proper vascular response and disruption
12 of autophagy can lead to a dysfunctional endothelium.
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29 **Endosomal signaling in the pulmonary endothelium in lung diseases**

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31 *Acute Respiratory Distress Syndrome*

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33 While studies demonstrate a role of the endosome in regulating the endothelium, less is
34 known about the role in the lung. We and others have demonstrated that members of the
35 endosomal cycling pathways, Rab GTPase family, Rab4, Rab5, Rab9 and Rab11, to play
36 a key role in regulating microvascular permeability and edema formation in both *in vitro*
37 and *in vivo* models of ARDS (11, 104, 105). Activation of the pro-recycling GTPases,
38 Rab4, and Rab11, and inhibition of pro-degradative GTPase, Rab9, elevates trafficking
39 of the junctional protein, vascular endothelial (VE)-cadherin, to the adherens junction,
40 thereby improving barrier integrity and reducing lipopolysaccharide (LPS)- and thrombin-
41 induced vascular leak (Figure 5) (11, 104). Conversely, inhibition of GTP-bound Rab5,
42 the GTPase associated with endosome internalization, attenuated LPS-induced barrier
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3 disruption (105) indicating the importance of these small GTPase family in regulating the
4 pulmonary microvasculature.
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10 *Pulmonary Hypertension*

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12 Recent studies have shown expression of the chloride intracellular channel 4 (CLIC4) to
13 be upregulated in the lungs of pulmonary arterial hypertension (PAH) patients and rodents
14 (102) and in endothelial colony forming units from PAH patients (1). Likewise, Arf6 was
15 also increased in endothelial colony forming units from PAH patients (1). CLIC4 localizes
16 to endocytic vesicles where it interacts with Arf G-proteins triggering the acidification of
17 the vesicles to lysosome and targeting BMPRII for degradation (1). As such, CLIC4 was
18 identified to regulate pulmonary endothelial cell function (81, 96) and targeted disruption
19 of CLIC4 or Arf6 expression was identified to attenuate PAH (1); studies suggesting.
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33 Other studies have shown the vasomotor tone of the pulmonary vasculature is regulated
34 via endocytosis of endothelial nitric oxide synthase (eNOS) *in vivo*. Caveolin-1 binding
35 of eNOS in the caveolae serves to inhibit its function (58). Upon endocytosis, caveolin-1
36 is phosphorylated, releasing eNOS from an inhibitory state promoting the synthesis of NO
37 (58). The vasodilatory response pulmonary vasculature in caveolin null mice is
38 attenuated (53). Heritable mutations of caveolin-1 predisposes individuals to developing
39 PAH (4). A recent study identified a frameshift mutation in the carboxyl terminus of
40 caveolin-1 disrupts its retention in the endoplasmic reticulum resulting in its degradation
41 and diminished caveolae formation (15). Thus, it is likely that development of pulmonary
42 hypertension is due in part to disrupted endocytosis of surface proteins, such as bone
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morphogenetic protein receptor II (BMPRII), eNOS or other proteins important in regulating the pulmonary vasculature.

Autophagic signaling in the pulmonary endothelium in lung diseases

Recent reviews have summarized evidence for regulated cell death and autophagy in lung diseases (2, 59, 79). In many lung diseases, oxidative stress alters protein folding, revealing amino acid motifs that are subsequently recognized by CMA machinery and targeted for autophagy, thereby restoring homeostasis. Additionally, mitochondrial injury may result in mitophagy and removal of damaged organelles. However, unchecked autophagy can also have deleterious effects, including excessive apoptosis and impaired angiogenesis. Table 1 summarizes the effects of various lung injuries on autophagy in lungs and endothelial cells and effects of autophagy on function. In general, autophagic processes are identified in lungs and cultured cells by increased expression of autophagy markers, such as increased ratio of microtubule-associated protein 1 light chain (LC3)-II to LC3-I and characteristic autophagosome morphology detected by transmission electron microscopy. Effects of autophagy on cell or organ function are generally inferred by the functional effects of autophagy inhibitors (e.g. 3-methyladenine), activators (e.g. rapamycin), or by altering expression of autophagy regulators (e.g. early growth response-1, Egr-1).

Pulmonary Hypertension

Pulmonary hypertension (PH) is characterized by vascular remodeling with abnormal proliferation of vascular cells, including endothelial cells, with increased intimal thickness and formation of plexiform lesions in pulmonary arterial hypertension. Studies in animal models have demonstrated initial endothelial apoptosis, followed by proliferation of apoptosis-resistant endothelial cells (75). Autophagy has been demonstrated in lungs of humans with pulmonary arterial hypertension and may serve a protective role against endothelial injury that initiates vascular remodeling in pulmonary hypertension (reviewed in (109)).

Lee *et al.* demonstrated increased expression of the autophagy marker, LC3B-II, in the endothelium of lungs from humans with PAH, with increased LC3B-II protein levels in lung tissue from PAH (IPAH) vs non-pulmonary hypertension controls, and in hypoxia induced PH in mice (45). Genetic downregulation of LC3B-II or early growth response-1 (Egr-1) (transcriptional regulator of LC3B-II) resulted in exacerbation of PH in mice and increased hypoxia-induced proliferation of endothelial and smooth muscle cells, suggesting that autophagy keeps vascular cell growth in check and may protect against PH development. Similarly, using the Sugen/ hypoxia model of severe PH in rats, Kato *et al* demonstrated increased expression of autophagy markers LC3 and beclin-1 in parallel with worsening pulmonary hypertension and endothelial cell proliferation (36). Rapamycin, an activator of autophagy, decreased PH and endothelial cell proliferation, with concomitant increase in endothelial cell autophagy and apoptosis, suggesting that endothelial cell autophagy suppressed the progression of endothelial cell proliferation resulting in diminished PH (36). Indeed, this is the case in methamphetamine-associated pulmonary arterial

hypertension, which occurs in individuals with a single nucleotide polymorphism that reduces expression of the methamphetamine metabolizing enzyme, carboxylesterase 1 (66). Pulmonary microvascular endothelial cells deficient in carboxylesterase 1 demonstrated reduced autophagic flux and increased apoptosis in response to methamphetamine. These results suggest that autophagy is protective against methamphetamine-induced pulmonary arterial hypertension.

However, others have demonstrated deleterious effects of autophagy in PH. Persistent pulmonary hypertension of the newborn is characterized by decreased lung blood vessel density. Teng *et al* reported increased expression of autophagy markers in pulmonary artery endothelial cells isolated from lungs of lambs with persistent pulmonary hypertension of the newborn (95). Inhibition of autophagy increased in vitro angiogenesis, suggesting that autophagy impairs angiogenesis in persistent pulmonary hypertension of the newborn.

In humans with HIV infection and in a simian model of HIV-associated PAH exposed to morphine, Dalvi *et al.* observed hyperproliferative and apoptosis-resistant endothelium (16) that expressed autophagy markers. Since opioid use increases the risk of developing PH in HIV-infected individuals, the authors assessed the effects of HIV-tat and morphine on cultured pulmonary endothelial cells. They noted increased autophagy flux and apoptosis in human pulmonary microvascular endothelial cells when treated with HIV-tat protein and morphine in a ROS-dependent manner. Autophagy inhibitors increased and activators diminished apoptosis. In addition, HIV-tat plus morphine-

induced increases in ROS were regulated by autophagy and autophagy inhibition increased ROS, suggesting that autophagy is regulating ROS. They concluded that autophagy of pulmonary endothelial cells may increase the extent of angioproliferative remodeling and thus the severity of HIV-associated pulmonary hypertension.

Similarly, Haslip *et al.* studied a mouse model of PH caused by intermittent hypoxia (28). They found that mice deficient in mitochondrial uncoupling protein 2 (UCP2) had more severe pulmonary hypertension after 5 weeks of intermittent hypoxia. Mouse lung endothelial cells deficient in UCP2 demonstrated increased mitophagy and apoptosis, similar to human pulmonary artery endothelial cells from humans with pulmonary arterial hypertension. They also concluded that endothelial cell autophagy was deleterious in pulmonary hypertension. Mao *et al* demonstrated that hypoxia-induced pulmonary vascular angiogenesis is dependent on increased autophagy (55).

Thus, whether autophagy is protective or deleterious in pulmonary hypertension depends upon whether it limits or increases abnormal endothelial proliferation or whether it prevents normal angiogenesis. More work is needed to determine the impact of autophagy on the pulmonary endothelial function in the setting of increased ROS and PH.

Acute Respiratory Distress Syndrome (ARDS)

ARDS is a syndrome of pulmonary edema, hypoxemia, and low lung compliance, frequently associated with inflammation and increased oxidant stress. Edema in ARDS is caused by increased pulmonary microvascular permeability resulting in water and protein

flux out of the vasculature and into the lung interstitium and alveolar gas space. Injury to the lung endothelium and epithelium are critical to increased permeability edema in ARDS.

Autophagy can be protective in ARDS caused by bacterial pneumonia by facilitating removal of pathogens by macrophages (xenophagy) (72). Similarly, autophagy can protect against sepsis-induced mitochondrial damage and downstream inflammasome activation and cytokine release, demonstrating a role for autophagy in regulating innate immune responses (64).

Hyperoxia (95% oxygen for 48-72 h) is a mouse model of severe oxidant lung injury characterized by inflammation, increased vascular permeability, and endothelial cell apoptosis. Silencing of lung endothelial hemoxygenase-1 (HO-1) increased inflammation, worsened survival, and increased endothelial apoptosis after hyperoxic exposure (113). Hyperoxia decreased expression of autophagy markers in both mouse lungs and cultured lung endothelial cells, an effect that was exacerbated by HO-1 suppression. These results indicate that hyperoxia decreases endothelial autophagy and suggest that HO-1 modulates this protective mechanism.

On the other hand, the lungs of humans and mice with H5N1 influenza infection demonstrated accumulation of autophagosomes and increased ratio of LC3B-II:LC3B-I autophagy marker expression (91). Studies of alveolar epithelial cells showed that H5N1 virus, but not H1N1, induced autophagy and decreased viability. Inhibition of autophagy

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3 by silencing Atg5 or by 3-methyladenine prolonged survival and decreased edema
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5 resulting from H5N1 pulmonary infection. Thus, in the case of lung injury caused by H5N1
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7 viral pneumonia, autophagy in epithelial cells appears to be deleterious.
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12 Similarly, recent studies indicate that autophagy enhanced LPS-induced endothelial
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14 barrier dysfunction. Zhang *et al.* reported that a chemical inhibitor of autophagy,
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16 chloroquine, and silencing of Atg7 blunted LPS-induced autophagosome formation in
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18 human pulmonary microvascular endothelial cells, decreased cell viability, and
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20 exacerbated LPS-induced monolayer permeability, (110). Similarly, Slavin *et al* reported
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22 that the autophagy inhibitor, 3-methyladenine (3-MA), reduced inhaled LPS-induced lung
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24 edema both when given prophylactically and as a treatment with minimal effects on
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26 inflammation and pro-inflammatory cytokine expression (83). In isolated endothelial cells,
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28 3-MA or silencing of Atg5 blunted thrombin-induced barrier dysfunction and reversed
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30 LPS-induced endothelial barrier dysfunction. Thus, autophagy appears to play a key role
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32 in promoting the breakdown of the endothelial barrier in response to edemagenic agents.
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40 The role(s) of autophagy in acute lung injuries resulting in ARDS are therefore complex
41
42 and dependent upon cell type studied and cause of lung injury. It has been proposed that
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44 therapeutic approaches to ARDS should not focus on autophagy alone, but on autophagy
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46 plus protection against oxidative stress, such as that conferred by Nrf2, a transcription
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48 factor that promotes genes that regulate oxidative stress (71).
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54 *Ischemia/Reperfusion Lung Injury*

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Ischemia/reperfusion lung injury can occur with pulmonary thromboembolism and lung transplantation and is characterized by lung inflammation and edema. Autophagy markers were increased in rodent lung models of ischemia/reperfusion injury (47, 112). Inhibition of autophagy by 3-methyladenine alleviated and rapamycin activation of autophagy aggravated lung injury. (47, 112) Pulmonary microvascular endothelial cells exposed to hypoxia, followed by reoxygenation, displayed increased mitophagy and apoptosis. (47). These results suggest a role for autophagy in endothelial injury caused by ischemia/reperfusion.

Emphysema

Emphysema is characterized by loss of alveolar/capillary septum and increased apoptosis of epithelial and endothelial cells (34, 35). Damaged organelles, such as mitochondria (59) and cilia (42), can be disposed of through autophagy (14) in chronic obstructive pulmonary disease. Mitophagy and ciliophagy have been demonstrated in bronchial epithelial cells. It is not clear to what extent mitophagy occurs in lung microvascular endothelial cells in emphysema.

With regard to the lung endothelium, we have reported that acute exposure to cigarette smoke increased lung vascular permeability and endothelial monolayer permeability through an oxidant-mediated effect (50). We also found that cigarette smoke extract increased cultured lung endothelial cell apoptosis and expression of markers of the unfolded protein response (eIF2 α phosphorylation) and autophagy (the increased ratio of LC3B-II:LC3B-I) (76). Using a mouse model of emphysema caused by intra-tracheal

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3 instillation of cadmium, Surolia et al demonstrated worsening of emphysema in HO-1
4 knock-out mice (92). Exposure of lung endothelial cells to cadmium increased both
5 apoptosis and expression of autophagy markers. Cadmium exposure of endothelial cells
6 from HO-1 knock-out mice increased apoptosis and decreased autophagy, while
7 overexpression of HO-1 or treatment with the autophagy activator, rapamycin, decreased
8 apoptosis and increased autophagy. These results indicate that HO-1 induces protective
9 autophagy in lung endothelial cells and is protective against cadmium-induced
10 emphysema.
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24 Table 1 demonstrates that autophagy, including endothelial cell autophagy, has been
25 demonstrated in lungs after various insults. However, the effects of autophagy on lung or
26 endothelial cell function--usually assessed by the effects of autophagy inhibitors or
27 activators or by suppression of key autophagy effectors—vary among injuries. Thus, there
28 is a need for additional study of the role of autophagy in lung diseases.
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INNOVATION:

Numerous diseases affecting the pulmonary endothelium, including emphysema, ARDS, and PAH, are linked to an imbalance in cellular homeostasis associated with altered endosomal or autophagy, leading to enhanced degradation of macromolecules and potential cell death. Efforts to target this imbalance have yielded limited success as a treatment for lung injuries, which is likely to be due to the complexity of processes involved. It is possible that novel therapeutic targets are the endosomal trafficking proteins, such as Rab GTPases and LAMTORs, as they also key players in the formation and maturation of the autophagolysosome. While regulating endocytosis or autophagy have been linked to improved function of the pulmonary endothelium *in vitro* and *in vivo*, further studies are vital to identify targets in modulating cellular homeostasis in the lung.

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AUTHOR DISCLOSURE STATEMENT

None of the authors have commercial associations or other financial conflicts to disclose.

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ABREVIATIONS:

AMP – adenosine monophosphate

AMPK – AMP kinase

ARDS - Acute Respiratory Distress Syndrome

Arf - ADP-ribosylation factor G proteins

Atg1 - autophagy-related genes-1

Bcl-2 – B-cell lymphoma 2

BMPRII – bone morphogenetic protein receptor II

clAP-1 - cellular inhibitor of apoptosis protein-1

CIE - clatherin independent endocytosis

CLIC4 - chloride intracellular channel 4

CMA - chaperone-mediated autophagy

CME - clatherin mediated endocytosis

EEA-1 – early endosome antigen 1

ELISA - enzyme-linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

ER - endoplasmic reticulum

ESCRTs - endosomal sorting complexes required for transport

FGFR1 - fibroblast growth factor receptor 1

GAP - guanine activating proteins

GDP – guanosine diphosphate

GEF - guanine exchange factors

GFP – green fluorescent protein

GTP – guanosine triphosphate

HO-1 - hemoxygenase-1

Hrs: hepatocyte growth factor-regulated tyrosine kinase substrate

HSC70 - heat shock cognate 71 kDa protein

JAM-A - junctional adhesion molecule-A

JIP4 - JNK-interacting protein-4

JNK-1 – c-Jun N-terminal kinase-1

LAMP2A – lysosomal associated membrane protein 2A

LAMTORS - Late endosomal/lysosomal adaptor, MAPK and MTOR activator 1

LC-3 - light chain-3

LPS - lipopolysaccharide

3-MA - 3-methyladenine

MEF – mouse embryonic fibroblasts

MKK - mitogen-activated protein kinase kinase

mTOR – mammalian target of rapamycin

MVB - multivesicular bodies

NO – nitric oxide

PAH - pulmonary arterial hypertension

PE - phosphatidylethanolamine

PTPN-13 - protein tyrosine phosphatase, non-receptor type 13

RCD - regulated cell death

RFP – red fluorescent protein

ROS – reactive oxygen species

RTK - receptor tyrosine kinases

SNAREs - soluble N-ethylamide-sensitive factor attachment protein receptors

TORC1 - mTOR complex 1

UCP2 - mitochondrial uncoupling protein 2

ULK1 - Unc-51 like autophagy activating kinase

VE-cadherin – vascular endothelial cadherin

VEGF - vascular endothelial growth factor

VEGFR2 – VEGF receptor 2

Vps34 - class III-type phosphatidylinositol 3 kinase complex

ZO - zonula occludens

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FIGURE LEGENDS:

Figure 1: *Schematic of endosome trafficking.* Trafficking of the endosome through the cell is regulated through a variety of specific proteins. The process of endocytosis is mediated by invagination of the plasma membrane at either clathrin-coated regions or caveolae. Once endocytosed, cargo is sorted in the early endosome before being either recycled back to the cell surface or trafficked to the late endosome for lysosomal degradation. The spatial and temporal control of this dynamic trafficking system is regulated by a range of protein families such as Rab-GTPases and ESCRTs. (Adapted from (69, 114)).

Figure 2: *Autophagic processes.* Schematic representation of chaperone-mediated autophagy, microautophagy, and macroautophagy. The three processes lead to the recycling of cellular building blocks and subsequent homeostasis.

Figure 3: *Physiological and pathological regulation of pulmonary circulation by endosomal and autophagic signaling.* Schematic representation of the intersection of endocytosis and autophagy and the effect on endothelial cell function.

Figure 4: *Cigarette smoke exposure increases markers of autophagy in pulmonary endothelial cells.* Pulmonary microvasculare endothelial cells were transfected with ptfLC3, which encodes LC3 fused to both RFP and GFP proteins; visualization via fluorescence microscopy of autophagic vacuoles (RFP⁺/GFP⁺; RFP⁺/GFP⁻),

autophagosomes (RFP⁺/GFP⁺), and autolysosomes (RFP⁺/GFP⁻). The endothelial cells were exposed to vehicle (panel a and d), cigarette smoke extract (panel b and d) for 2 hours, or serum deprivation as a positive control for 30 mins (panel c). Panels a-c: immunofluorescence of RFP and GFP was used to assess the level of RFP-positive autophagic vesicles. Yellow arrows indicate co-expression of RFP and GFP indicative of autophagosomes whilst white arrows show RFP staining which is indicative of autophagolysosomes. Panel d: Expression of LC3I and II was assessed by Western blotting with increased LC3II indicative of autophagy. Unpublished data.

Figure 5: *Endosome regulators are both vascular protective and vascular disruptive.*

Panel a: Pulmonary microvascular endothelial cells were transiently transfected with dominant active Rab 4 (Rab4^{Q67L}), dominant negative Rab 9 (Rab9^{S21N}), Rab 7 (Rab7^{T22N}) constructs, or a green fluorescent protein (GFP) vector control, and exposed to lipopolysaccharide (LPS) for 6 hours. Panel a, (i) whole-cell ELISA was used to assess the cell-surface expression of the adherens junction protein, VE-cadherin and (ii) electric cell impedance system was used to measure the permeability as the drop in endothelial monolayer resistance. n=6, *p<0.05 versus vehicle for LPS (adapted from (11)) Panel b: a schematic figure representing the key vascular protective and vascular disruptive endosomal regulators.

Table 1: *Autophagy in Lung Diseases.* Summary of literature demonstrating autophagy, including endothelial cell autophagy, has been demonstrated in lungs after various insults.

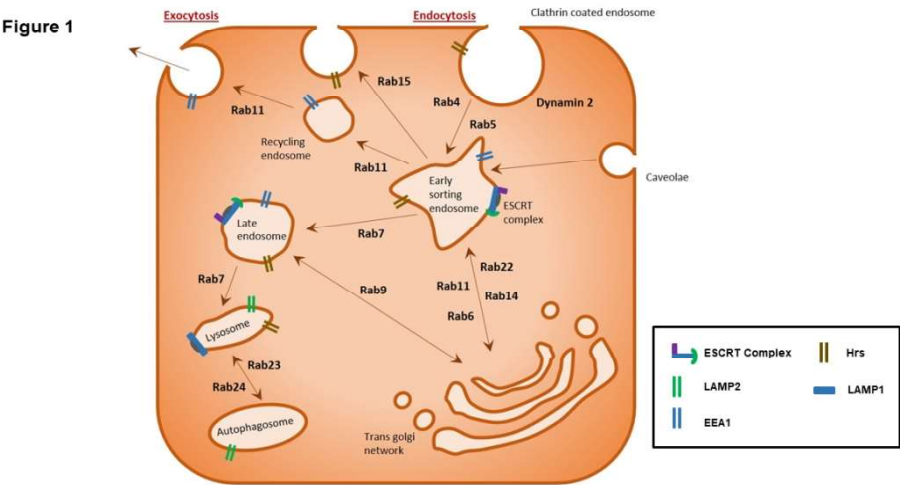


Figure 1

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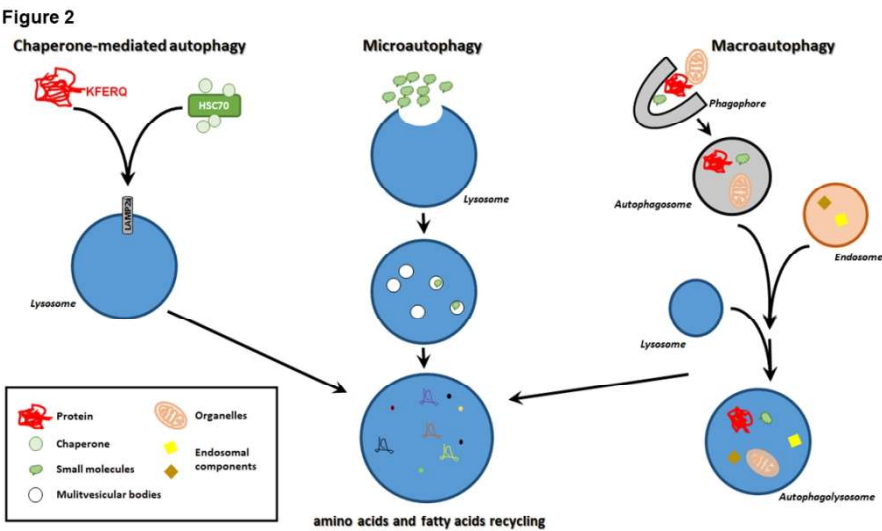


Figure 2

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Figure 3

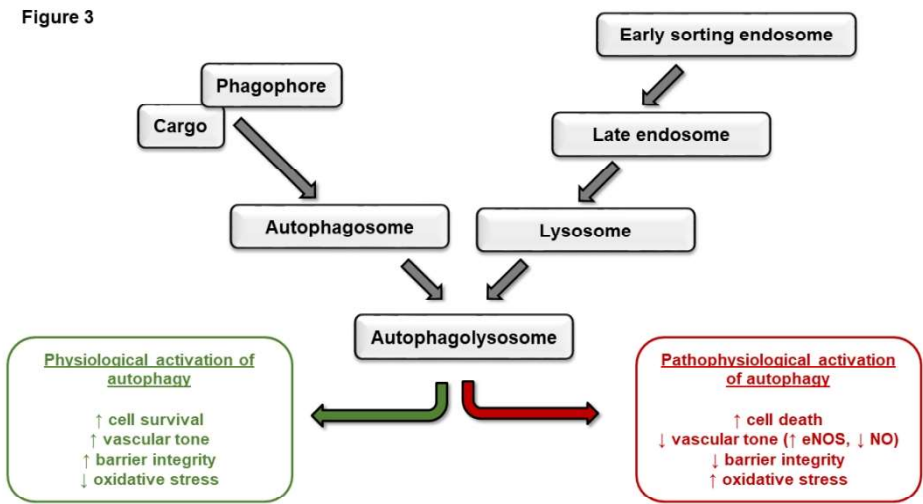


Figure 3

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Figure 4

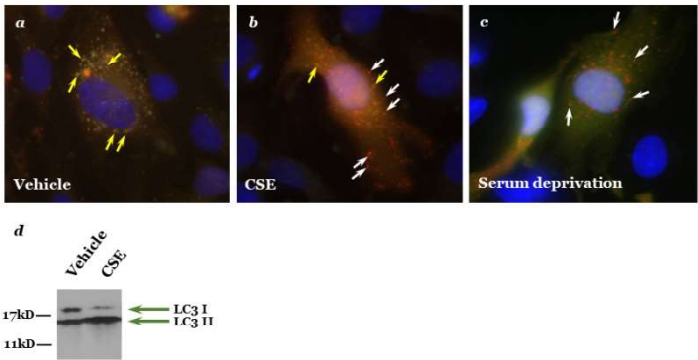


Figure 4

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Figure 5

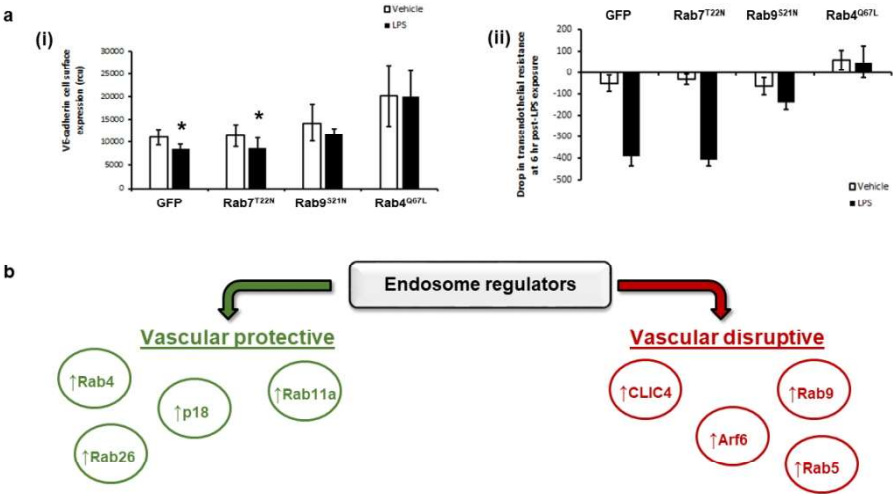


Figure 5

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Table 1: Autophagy in Lung Diseases

Lung Disease	Cause	Autophagy Marker in Lung	Autophagy Marker in Lung Endothelial Cells	Effect of Autophagy on Function	Reference
Pulmonary Hypertension	Idiopathic PAH	↑	ND	Improve	92
	Hypoxia	↑	↑	Improve	92
	Sugen-hypoxia	↑	↑	Improve	93
	Methamphetamine	ND	↑	Improve	94
	Persistent PH of Newborn	↑	↑	Worsen	95
	HIV-tat/Morphine	↑	↑	ND	96
	Hypoxia/Ucp2 Knock Out	Mitophagy ↑	↑	Worsen	97
	Bacterial Pneumonia	Xenophagy ↑	ND	Improve	99
ARDS	Sepsis	ND	ND	Improve	100
	Hyperoxia	↑	↑	Worsen	101
	HSN1 Infection	↑	ND	Worsen	102
	Endotoxin	ND	↑	Improve	103
	Endotoxin	ND	ND	Improve	104
	Cigarette Smoke	ND	↑	ND	113
	PA occlusion	↑	ND	Worsen	107
Ischemia/reperfusion	Lung Transplant	Mitophagy ↑	↑	Worsen	106
Emphysema	Cigarette Smoke	Mitophagy ↑	ND	Worsen	88
	Cigarette Smoke	Cliophagy ↑	ND	Worsen	110
	Cadmium	ND	↑	Improve	114

N.D. = Not Done

Table 1

338x190mm (96 x 96 DPI)