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Implications of autophagy for vascular smooth muscle cell function and plasticity

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Abstract

Vascular smooth muscle cells (VSMCs) are fundamental in regulating blood pressure and distributing oxygen and nutrients to peripheral tissues. They also possess remarkable plasticity, with the capacity to switch to synthetic, macrophage-like or osteochondrogenic phenotypes when cued by external stimuli. In arterial diseases such as atherosclerosis and restenosis, this plasticity appears to be critical, and depending on the disease context, can be deleterious or beneficial. Therefore, understanding the mechanisms regulating VSMC phenotype and survival are essential for developing new therapies for vascular disease as well as understanding how secondary complications develop due to surgical interventions. In this regard, the cellular process of autophagy is increasingly being recognized as a major player in vascular biology and a critical determinant of VSMC phenotype and survival. Although autophagy was identified in lesional VSMCs in the 1960s, our understanding of the implications of autophagy in arterial diseases and the stimuli promoting its activation in VSMCs are only now being elucidated. In this review, we highlight the evidence for autophagy occurring in VSMCs *in vivo*, elaborate on the stimuli and processes regulating autophagy, and discuss the current understanding of the role of VSMC autophagy in vascular disease.

Keywords

atherosclerosis; restenosis; hypertension; oxidative stress; cardiovascular; proliferation

INTRODUCTION

Vascular smooth muscle cells (VSMCs) are primary constituents of the blood vessel wall. In resistance vessels, they help maintain vascular tone, thereby regulating blood pressure and the distribution of oxygen and nutrients to peripheral tissues. The physiologic regulation of

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vascular function derives largely from the well-studied collaboration between VSMCs and endothelial cells. Such cooperative functionality of resistance vessels is commonly lost in diseases such as atherosclerosis and hypertension or with physical disruption of endothelial cell-VSMC communication during percutaneous coronary interventions [1–3].

The environmental changes caused by disease or surgical interventions result in the exposure of VSMCs to cytokines produced by inflammatory cells as well as to growth factors, lipids, and reactive products derived from oxidative stress. Depending on the disease context, the response of VSMCs to these insults or stimuli is viewed as beneficial or deleterious. For example, VSMCs could contribute to atheroma formation by converting from a contractile phenotype to a macrophage-like phenotype, capable of taking up modified low-density lipoproteins, free cholesterol, or apoptotic cells. Conversely, in advanced atheromas, VSMCs commonly assume a synthetic phenotype with high proliferation capacity, migratory prowess, and enhanced production of extracellular matrix (ECM) [1, 2]. In this scenario, VSMCs are thought to increase plaque stability, thereby preventing rupture and atherothrombosis [4–7].

Phenotype switching has been shown to be a significant contributor to other diseases as well. A switch to the synthetic VSMC phenotype occurs in the vessel wall in response to mechanical injury, such as occurs with vessel stenting or grafting, and this can result in aggressive neointimal VSMC proliferation and restenosis [1, 8, 9]. Changes in VSMC phenotype, resulting in impaired contractility and vascular calcification, also occur in hypertension and with increasing age, and loss of VSMCs due to apoptosis is thought to contribute to the development of aneurysms [1]. In diabetes, changes in VSMC phenotype associated with loss of glucose control and metabolic disease may contribute to the progression of vasculopathies [10]. Therefore, understanding the mechanisms that regulate VSMC phenotype and function is important for developing novel therapies to treat vascular disease.

Accumulating evidence suggests that autophagy is activated in VSMCs in vascular disease and that it is important for VSMC survival and plasticity. While multiple studies have shown autophagy to be important in other cell types or processes in cardiovascular disease (e.g., heart failure [11]), this review focuses specifically on the role of autophagy in VSMCs.

GENERAL FUNCTION AND REGULATION OF AUTOPHAGY

The phenomenon of autophagy (and mitophagy) appears to have been first identified in electron micrographs by Clark in 1957 [12], where it was described that differentiating cells in the newborn mouse kidney show “bodies and vacuoles...surrounded by dense membranes” that “contain canalicular structures, dense lamellar inclusions, and altered mitochondria.” Dr. Clark concludes that his observations “are consistent with the hypothesis that the large bodies represent vacuoles which have accumulated a high concentration of amorphous material” [12]. Several years later, Christian De Duve first used the term “autophagy” to describe this process [13, 14] and was later awarded the Nobel Prize for describing the structure and function of organelles such as lysosomes. More than half a century later, we continue to study autophagy and lysosomal processes and how they regulate (patho)physiology.

It has become clear that autophagy is a process used for cellular renovation—a “self-eating” employed by the cell for the recycling of cytoplasmic contents—and is one of two major degradation systems in eukaryotic cells. Whereas the proteasome follows a relatively simple archetype exemplified by degradation of primarily short-lived, ubiquitinated substrates [15, 16], autophagy is much more complex, involving lysosomal degradation of cytosolic components, extracellular material and plasma membrane proteins [17–20]. There are

generally three classes of autophagy—macroautophagy, microautophagy, and chaperone-mediated autophagy—and although each of these forms utilize the lysosome to degrade intracellular components, the route or mechanism of cargo delivery differs. Macroautophagy is thought to be the major form of autophagy and herein will be referred to as “autophagy.” Autophagy also consists of several selective forms that involve the degradation of different intracellular materials. For example, aggrephagy, mitophagy, pexophagy, reticulophagy, ribophagy and xenophagy are selective pathways for the degradation of aggresomes, mitochondria, peroxisomes, endoplasmic reticulum, ribosomes and bacteria and viruses, respectively. The reader is directed to several excellent reviews on various classes and forms of autophagy [17–19, 21, 22].

Autophagy is a highly inducible and finely controlled cellular event. Classic stimuli such as starvation, oxidative stress, and infection result in the activation of cell signaling pathways and regulatory proteins and complexes that precipitate autophagosomal membrane nucleation, elongation, and maturation (Fig. 1). Autophagosomes generally form near the endoplasmic reticulum (ER), and several studies suggest that membranes derived from mitochondria, the Golgi apparatus, and the plasma membrane also could contribute to autophagosome formation [23]. Most recently, it was shown that the autophagosome forms at contact sites between the ER and mitochondria [24, 25].

The molecular machinery governing the formation of autophagosomes was first described in yeast, and many of the genes involved have mammalian orthologs [22]. Required for formation are the “core autophagy-related (Atg) proteins”—Atg1–10, 12–14, 16 and 18 as well as Atg17, 29, and 31. The principle process is initiated by activation of the Atg1 complex [also called the Unc-51-like kinase (ULK) complex], which in mammals is composed of the Atg1 homolog ULK 1 or 2, Atg13, FAK-family interacting protein of 200 kDa (FIP200, a counterpart of yeast Atg17), and Atg101 (an Atg13-binding protein). Nutrient-related signals or growth factors that stimulate the class I, phosphatidylinositol 3-kinase (PI3K)-Akt pathway result in mammalian target of rapamycin complex I (mTORC1)-mediated repression (inactivation) of the Atg1 (ULK) complex. Conversely, conditions of energy deprivation inhibit mTORC1, which permits activation of the Atg1 complex [17, 26]. Activation of Atg1 leads to stimulation of the Beclin 1–PI3KIII complex. This complex serves as a regulatory platform and generates phosphatidylinositol-3-phosphate, which is required for nucleation. Elongation and maturation requires the Atg5-Atg15 and microtubule-associated protein light chain 3 (LC3) conjugation systems. The latter conjugation reaction involves the conjugation of phosphatidylethanolamine (PE) to microtubule-associated protein light chain 3-I (LC3-I) to form LC3-II, which stabilizes the growing autophagosome. Formation of LC3-II is typically used as an indication of autophagic activation [27, 28]; by immunofluorescence analysis, it is visible as discrete puncta, and by Western blot analysis, it is discerned by a lower apparent molecular mass form of LC3 (~14 kDa). Once the autophagosome has matured, it fuses with the lysosome to form an autolysosome equipped with acid hydrolases which degrade intra-autolysosomal contents [22] (Fig. 1).

AUTOPHAGY IN VSMCS

Realization of the importance of autophagy in VSMCs has only recently emerged. In the following sections, we pose several critical questions that are directed to help understand the role of autophagy in VSMCs and guide future studies. Each question is answered in turn based on past and current literature.

What is the evidence for autophagy occurring specifically in VSMCs *in vivo*?

While multiple studies document the occurrence of autophagy in cardiovascular tissues [19, 29–34], comparatively less has been reported regarding autophagy occurring in vascular smooth muscle *in vivo* (Table 1). Electron microscopic (EM) studies from the early 1960s provided the first evidence of autophagy occurring in VSMCs in cardiovascular disease. The first report in humans was published in 1961, where “cytoplasmic inclusions containing... dense material... limited by a double membrane” were found in atherosclerotic lesions [35]. Interestingly, the authors found that these “inclusions were found most often in smooth muscle, but were occasionally present in foam cells” [35]. Similarly, aortas from rabbits fed a high cholesterol diet followed by reversion to normal chow showed “modified smooth muscle cells” in the atheromas. These VSMCs were described as the predominant cells in the subendothelial tissue and many of them contained “large, very dense vacuoles” containing “either haemosiderin or osmiophilic liquid” [36]. Haemosiderin is insoluble, non-heme, cytoplasmic iron that is thought to derive from ferritin after autophagy [37–42]. That these vacuoles likely contained haemosiderin thus suggests that the author was observing autophagic vacuoles. Multiple later studies in humans, baboons, monkeys, rabbits, and rats showed convincing EM evidence of autophagy in VSMCs occurring in the context of atherosclerosis and hypertension [43–50].

The more recent understanding of the molecular regulation of autophagy has also led to identification of autophagy occurring in VSMCs in diseased vessels. For example, a monocrotaline-induced model of pulmonary hypertension resulted in loss of p62 in medial pulmonary artery SMCs that was partially restored by treatment with the autophagy inhibitor, chloroquine [51]. Surprisingly, however, the advent of new techniques for monitoring autophagy, such as the analysis of GFP-LC3 (Atg8) in transgenic mice [52, 53] or direct detection of LC3 [54, 55] or granular cytoplasmic ubiquitin inclusions [56–59] by immunohistochemistry, has not further increased our awareness of the occurrence and roles of autophagy in VSMCs in diseased vasculature *in vivo*. This is likely due to several limitations. First, current models used for studying atherosclerosis may not reflect the human disease. ApoE^{-/-} and LDLR^{-/-} mice are the most well-described animal models [60–62], and while these mice develop advanced lesions relatively quickly, the lesions contain notoriously less VSMCs than lesions and atheromas from humans and other animal models of atherosclerosis such as hypercholesterolemic rabbits [63]. Hence, while development of a VSMC-specific knockout of important autophagic genes such as Atg5 would likely be helpful in understanding the role of VSMC autophagy in restenosis, its value in atherosclerosis is less clear. Clearly, transgenic animal models that more closely resemble human disease might prove useful for monitoring and studying the role of autophagy in VSMCs in atherosclerosis.

Second to this lower abundance of VSMCs in atherosclerotic plaques of mice are several technical issues. Atheromas commonly show strong fluorescence background [64], which could interfere with imaging of fluorescently labeled LC3. Furthermore, data from immunohistochemical imaging of native LC3 or ubiquitinated aggregates may be difficult to interpret due to limitations in the abundance of the LC3-II protein [64] or to uncertainty of whether ubiquitin inclusions are due to increased autophagic flux [65].

What stimuli have been shown to activate autophagy in VSMCs?

Much of our knowledge of the mechanisms and stimuli that activate autophagy in VSMCs derive from experiments using cultured cells. Factors related to the disease process, such as reactive species derived from oxidative stress, inflammatory signaling, mitogens, metabolic stressors, and specific pathways regulating cellular homeostasis (e.g., ER stress) have been shown to promote VSMC autophagy and are discussed below.

Activation of autophagy by reactive species—As with many other cell types, the induction of autophagy in VSMCs is indirectly or directly related to the production of reactive oxygen or nitrogen species (ROS/RNS). Likely most important of these species are O_2^- and H_2O_2 because they both are formed by controlled mechanisms and can participate in redox signaling processes. The intracellular sources of these species include the mitochondrial electron transport chain [66], NADPH oxidases [67], peroxisomes [68] and the cytochrome P450 system [69]. How these sources of oxidants regulate autophagy has been reviewed elsewhere [70]; however, it is worth noting that critical components of the autophagic pathway such as thiol residues of Atg4 can be modified directly by ROS such as H_2O_2 [71], thereby integrating redox tone and signaling with induction of the autophagic process.

In VSMCs, multiple studies implicate “oxidative stress” in the activation of autophagy, with “oxidative stress” meant here simply as an increase in oxidants over control conditions. An *in vitro* model of phosphate-induced vascular calcification showed that addition of inorganic phosphate (P_i) to bovine aortic SMCs resulted in increased autophagy, which was shown to be due to P_i -mediated increases in the production of mitochondrial-derived superoxide. Interestingly, inhibition of autophagy increased calcification by increasing phosphate (P_i)-induced matrix vesicle (MV) release from VSMCs whereas activation of autophagy with valproic acid decreased calcification, implying that autophagy plays a protective role in this context [72]. Nonetheless, it is important to note that generalized increases in ROS do not always promote autophagy in VSMCs. For example, overexpression of apolipoprotein L6 (ApoL6) in VSMC-like cells derived from the fibrous cap of human atherosclerotic lesions resulted in increased ROS generation and apoptosis when stimulated with interferon- γ ; yet ApoL6 inhibited autophagy [73]. Clearly, in this context, ROS were not sufficient to induce autophagy.

Although ROS have been shown to be capable of promoting autophagy in VSMCs [72] as well as other cell types [70, 74–76], secondary products of oxidative stress such as oxidized lipids have largely been implicated in the activation of autophagy. The generation of these products is typically initiated by radical reactions with polyunsaturated fatty acids, and continued propagation of peroxidation reactions result in the formation of reactive lipid species such as free aldehydes (e.g., 4-hydroxynonenal; HNE) which can covalently modify proteins, affecting their structure and/or function [77]. In addition, bioactive core aldehydes such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), which remain esterified to the glycerol backbone of phospholipids, are also formed in such reactions [78]. Reactive lipids such as these have been shown to accumulate in diseased vessels in arterial restenosis [79], vasculitis [80], and atherosclerosis [81–83] and have been related with increases in VSMC proliferation, phenotype switching, or apoptosis (*vide infra*).

Both HNE and POVPC, as well as the highly electrophilic aldehyde—acrolein, were shown to activate autophagy in VSMCs [84]. The degree of autophagic activation due to such products appears to depend upon their electrophilicity: the non-electrophilic C9 analog of HNE, nonanal, did not induce autophagy; the mildly electrophilic analog nonenal promoted modest LC3-II conversion; and the highly electrophilic lipids HNE and acrolein increased autophagy significantly [84]. The activation by reactive lipids appears to promote VSMC survival by removing proteins damaged by the oxidized lipids; inhibition of autophagy with 3-methyladenine (3-MA) leads to stabilization of protein-HNE adducts and promotes cell death [84].

Mechanistically, autophagy is activated by oxidized lipids in VSMCs in an endoplasmic reticulum (ER) stress-dependent manner. HNE was shown to promote the carbonylation of

the ER-resident proteins protein disulfide isomerase (PDI), glucose regulated protein 78 (Grp78), Grp58, and reticulocalbin and to cause selective phosphorylation of protein kinase R-like ER kinase (PERK) and eIF2 α of the unfolded protein response (UPR) and activate the stress kinases p38 and JNK [85]. Inhibition of ER stress with the chemical chaperone 4-phenylbutyric acid (PBA) prevented activation of JNK and autophagy by HNE, and inhibition of JNK was sufficient to prevent HNE-induced autophagy. Hence, it appears that autophagic responses triggered by unsaturated aldehydes promote cell survival and are attributed, in part, to ER stress, which stimulates autophagy by a JNK-dependent mechanism [85]. How JNK activates autophagy in VSMCs is unclear; but, in cancer cells, JNK activation has been shown to upregulate Beclin 1, which was implicated in autophagic cell death [86, 87]. Whether this JNK-mediated mechanism underlies HNE-induced autophagy in VSMCs remains to be elucidated.

Reactive lipids such as HNE are known to be released from the oxidation of low density lipoprotein (LDL) [88–91]. HNE is also found to covalently modify oxidized LDL (oxLDL), which increases the atherogenicity of the particle [92–94]. Although multiple studies show oxLDL-mediated increases in autophagy in other vascular cells, e.g. endothelial cells [95–99], there are few reports of autophagy induced by oxLDL in VSMCs. Exposure of VSMCs to relatively modest concentrations of oxLDL (10–40 μ g/ml) was shown to increase autophagy, whereas high concentrations (60 mg/dl) resulted in decreases in autophagy and increases in apoptosis [100]. Interestingly, in that study, the miRNA hsa-let-7g was shown to inhibit oxLDL-induced autophagy by diminishing lectin-like oxLDL receptor (LOX-1) expression [100]. In pericytes—which are VSMC-like, peri-endothelial support cells important in angiogenesis and arteriogenesis [101–104]—highly oxidized and glycated LDL (HOG-LDL) was shown to increase autophagy as well as promote ER stress, mitochondrial dysfunction, and apoptosis [105].

Like HNE, one of the primary cytotoxins in oxLDL—the oxysterol 7-ketocholesterol (7-KC)—is also a potent activator of autophagy in VSMCs. Treatment of human VSMCs with 7-KC resulted in increased protein ubiquitination, autophagy and cell death [106]. However, similar to HNE-induced autophagy [84], 7-KC appears to promote a protective form of autophagy, as VSMC death induced by low concentrations of statins was decreased when 7-KC was present [107]. Furthermore, a recent study showed that 7-KC-induced autophagy is primarily protective and could be mediated by ROS [108]. Administration of 7-KC to VSMCs promoted upregulation of NADPH oxidase 4 (Nox4) and increased H₂O₂ production, which resulted in inhibition of Atg4B activity. Overexpression of catalase or knockdown of Nox4 inhibited 7-KC-induced autophagy, and inhibition of 7-KC-mediated autophagy promoted cell death, whereas further activation of autophagy with rapamycin in 7-KC-treated cells promoted survival [108]. Hence, 7-KC may modulate autophagy by promoting ROS-mediated regulation of the autophagic machinery.

Exactly how autophagy suppresses cell death programs are unclear, but it has been proposed that autophagy could remove defective mitochondria, thereby limiting the release of pro-apoptotic factors such as cytochrome *c* and apoptosis-inducing factor [109, 110]. Furthermore, the study by He and colleagues showed that activation of autophagy in VSMCs attenuates 7-KC-induced ER stress [108]. That both 7-KC and HNE promote a protective form of autophagy are consistent and may be due to an identical mechanism: oxLDL and 7-KC treatment both lead to increases in protein-HNE adducts and ER stress [82, 106, 111, 112], which may ultimately be the trigger for this form of autophagy [84, 85]. These studies suggest that ER stress and autophagy are highly integrated and play essential homeostatic roles in the cell. Consistent with this notion, VSMCs and macrophages in human atheromas show increased ER stress and abundance of 7-KC and/or HNE-modified proteins [111, 112]. Importantly, free, unoxidized cholesterol was also shown to increase a

protective form autophagy in VSMCs [113]. Whether similar mechanisms regulate the induction of autophagy by non-oxidized cholesterol in VSMCs remains to be determined.

While induction of autophagy by oxLDL may be due to species such as HNE or 7-KC, the role for protein glycation is less clear; however, it does appear that advanced glycation end products (AGEs) induce autophagy by a mechanism distinct from reactive lipids. VSMCs treated with AGEs resulted in induction of autophagy, which was associated with activation of ERK, JNK and p38 and inhibition of Akt. Inhibition of ERK, activation of Akt, and knockdown of the receptor of AGEs (RAGE) inhibited AGE-induced autophagy [114]. Hence, receptor signaling through RAGE likely regulates AGE-mediated autophagy in VSMCs, while oxidized lipids and perhaps similar reactive species form adducts with intracellular proteins, which triggers ER stress and JNK activation, leading to autophagy. A major challenge now is to better understand how each of these reactive species integrate in the context of disease to regulate VSMC function and arterial disease.

Induction of autophagy by cytokines and growth factors—Autophagy is known to be differentially regulated by numerous cytokines and growth factors [115]. In VSMCs, TNF α , which is present in atheromas and can be secreted by inflammatory cells as well as VSMCs [116, 117], was shown to increase cell death, autophagy and Beclin 1 expression [118]. TNF α -induced autophagy was associated with JNK activation and inhibition of Akt. In the same study, the authors found that insulin-like growth factor-1 (IGF-1)—known to be downregulated by TNF [119]—inhibited the formation of autophagosomes and increased Akt phosphorylation [118].

Other cytokines such as osteopontin (OPN) have been shown to be potent stimulators of autophagy in VSMCs. OPN was implicated in the induction of autophagy, leading to VSMC death in abdominal aortic aneurysm (AAA) [120]. Interestingly, the levels of key autophagic genes encoding LC3, Atg4b, Beclin1/Atg6, Bnip3 and Vps34 were markedly upregulated in AAA tissues. These changes were associated with autophagy and a remarkable increase in OPN abundance. A causal role for OPN in autophagy occurring in AAA was suggested by studies showing that recombinant OPN was sufficient to induce autophagy and cell death of VSMCs. Interestingly, while OPN stimulated multiple kinases, including JNK, only inhibition of the p38 pathway inhibited OPN-induced autophagy. Because OPN-induced autophagy could also be blocked with an integrin inhibitor or CD44 antibody, the authors conclude that OPN-induced autophagy proceeds via integrin/CD44 and p38 signaling [120].

Platelet-derived growth factor (PDGF), which can be secreted from platelets as well as endothelial cells and macrophages [121], is well known to stimulate VSMC proliferation and migration and is posited to be a major player in restenosis and atherosclerosis. Our recent study showed that PDGF induces a unique form of autophagy in VSMCs that regulates phenotype transition [122]. Cells treated with PDGF-BB showed a robust increase in autophagy that was associated with attainment of the synthetic phenotype, which is typically characterized by loss of contractile proteins such as α -smooth muscle actin and calponin and increases in the abundance of collagen-I and OPN. Inhibition of PDGF-induced autophagy with spautin-1 or 3-MA prevented loss of the contractile phenotype. It is posited that activation of the autophagic program by PDGF results in enhanced degradation of contractile proteins, thereby allowing VSMCs to rapidly reach the synthetic state. This concept is further supported by data showing that bafilomycin, which inhibits the acidification of autophagosomes required for protein hydrolysis, stabilized calponin levels in PDGF-treated cells. Like HNE-induced autophagy, autophagy caused by PDGF treatment enhanced the degradation of proteins damaged by electrophilic lipids [122].

That this form of autophagy promotes proliferation is consistent with a recent study showing that 3-MA prevents arterial restenosis [123]. In that study, the secreted protein Sonic hedgehog (Shh), which is critical in organ development [124] and regulates VSMC growth [125] and angiogenesis [126, 127], increased autophagy in mouse aortic SMCs in an Akt-dependent manner. Like PDGF-induced autophagy [122], Shh-mediated autophagy appeared to support proliferation, suggesting that autophagy may be important to the development or maintenance of the hyperproliferative VSMC phenotype. Whether there is a direct link between Shh- and PDGF-mediated autophagy is presently unclear; however, miRNA 140, which negatively regulates PDGF signaling during development, was shown to decrease Shh expression [128]. Hence, it is highly likely that secreted factors may integrate to promote autophagic responses. In further support of this, PDGF is well known to stimulate OPN [122, 129–131], which can promote autophagy in VSMCs [120]. Whether Shh, PDGF, and OPN integrate to promote growth factor-mediated autophagy, or whether these factors by themselves are sufficient to induce autophagy is unclear.

Metabolic stress—While starvation and nutrient stress are known to be activators of autophagy in multiple cells and tissues [17–19], there is relatively little known about how metabolic stress impacts VSMCs *in vivo*. Similar to data showing an increase in autophagy in adipose tissues in the context of obesity [132], nutrient excess in mice promotes autophagy in vascular tissues [133]. Feeding a high fat diet to mice for 8 weeks resulted in a decrease in p62 and increased Atg7 and LC3-II expression in aorta, and electron micrographs showed a twofold increase in autolysosome-like vacuoles in the vascular tissues of obese mice. This increase in autophagy was associated with insulin resistance and an increase in the phosphorylation of PERK and eIF2 α . Autophagy and the nutrient excess-driven phenotype were reversed by administration of osteocalcin [133], an emerging regulator of glucose and lipid metabolism secreted by osteoblasts [134–136]. In cultured VSMCs, osteocalcin reversed autophagy, ER stress (PERK and eIF2 α), and insulin resistance in cells treated with the UPR-inducer tunicamycin. Similar effects of osteocalcin were observed in cells with decreased expression of XBP-1 or knockout of Atg7. Inhibition of Akt, mTOR or NF- κ B nullified the protective effects of osteocalcin [133].

While the mechanisms of how diet composition and nutrient excess regulate autophagy in VSMCs remain to be further elucidated, it is likely that insulin signaling plays an important role. Both insulin and the closely related peptide insulin-like growth factor-1 (IGF-1) are known to activate the PI3K/Akt/mTOR pathway, resulting in inhibition of autophagy [137–141]. Overindulgence in calorie-rich foods is an instigating factor in metabolic syndrome, and this typically results in heightened plasma levels of insulin and the development of insulin resistance in peripheral tissues. While such changes have been shown to be associated with vascular disease [142], the role of insulin-modulated autophagy in VSMCs remains unclear. In VSMCs, insulin and IGF-1 have the capacity to diminish autophagy [84, 118], and our unpublished observations suggest opposing effects of insulin on PDGF-induced autophagy. Nevertheless, how insulin signaling regulates VSMC autophagy, function and metabolism, and whether such changes impact VSMC autophagy *in vivo* remain to be determined.

Hypoxia, a metabolic stressor in its own right, was also shown to promote autophagy in human pulmonary VSMCs [143]. Interestingly, the metabolic sensor AMPK appears to play an important role in the VSMC autophagy caused by hypoxia. AMPK was shown to be phosphorylated and activated in pulmonary VSMCs isolated from both pulmonary artery hypertensive patients and hypertensive mice, and inhibition of AMPK α 1 prevented hypoxia-induced autophagy, causing cell death. Furthermore, the AMPK inhibitor compound C reversed hypoxia-induced pulmonary hypertension [143]. Hence, AMPK

appears to play a key role in autophagy and the survival of pulmonary artery SMCs during hypoxia.

What is the role of autophagy in VSMC phenotype and vascular disease?

VSMCs are a phenotypically heterogeneous population in the arterial wall and not only maintain a remarkable ability to dedifferentiate into the synthetic phenotype, but to switch to macrophage-like and osteochondrogenic cell types as well. More than 50 years of evidence appear to support the hypothesis that VSMCs dedifferentiate in response to arterial damage, such as occurs after percutaneous interventions or during atherosclerosis. This process generally results in a highly proliferative VSMC phenotype capable of laying down extracellular matrix and recruiting inflammatory cells [1, 2].

While this concept—i.e., that medial VSMCs are major contributors to neointimal lesions—has been recently challenged [144], it does appear that the dedifferentiation hypothesis currently holds greater support [145]. For example, Nemenoff et al used lineage tracing studies to provide strong evidence that differentiated VSMCs, after wire injury of the femoral artery, undergo a phenotype switch and contribute the majority of neointimal cells in restenosis [146]. The conclusions of this study are backed by multiple atherosclerosis and restenosis studies that provide compelling evidence showing large numbers of medial VSMCs capable of multiple rounds of cell division [147–150]. Indeed, much older studies suggest that VSMCs dedifferentiate and migrate to the neointima. For example, electron microscopic studies of ligated arteries by Robert C. Buck showed “dedifferentiated smooth muscle cells, which first appeared in so-called ‘splits’ in the internal elastic lamina, [that] could be traced to the inner muscle compartment of the media through fenestrations in the elastic lamina.” Interestingly, Buck comments on the features of the cells and notes that they “differed from smooth muscle of arterial media: the fibrils, even 10 months after operation, never filled the greater part of the cytoplasm....” and “dense bodies, apparently representing phagocytic vacuoles, were present..” [151]. Hence, this perhaps is another example of an early report of autophagy that coincides with the de-differentiated phenotype of VSMCs.

Many of the autophagy-inducing stimuli discussed above promote both loss of the contractile phenotype and increase VSMC proliferation. For example, both PDGF-BB and POVPC promote loss of contractile gene expression and increase cell proliferation and migration [152–157] as well as induce autophagy [84, 122]. Similarly, OPN results in both autophagy and a more proliferative phenotype [120, 158, 159]. In the case of PDGF, inhibition of autophagy resulted in maintenance of the contractile phenotype and prevented hyperproliferation [122]. That autophagy in these contexts may play a role in cell division is adduced by data showing that inhibition of autophagy prevents cell proliferation caused by PDGF [122] and Shh [123]. Hence, it appears that some growth factors and oxidized lipids promote a unique form of autophagy that has the capacity to hasten the development of the synthetic phenotype and promote a hyperproliferative phenotype. How this form of autophagy could integrate with the signaling pathways and transcriptional programs known to trigger the synthetic phenotype, e.g., myocardin, Kruppel-like factor 4, Elk-1, miRs, etc. [1, 2, 104, 160], remains to be elucidated.

In contrast, other inducers of autophagy have been shown to prevent VSMC phenotype switching and hyperproliferation. For example, rapamycin-based drugs (e.g., sirolimus and everolimus) commonly used in drug-eluting stents are used to prevent restenosis following angioplasty, and these drugs are known inhibitors of the mTOR pathway and inducers of autophagy [161]. Unlike growth factors and oxidized lipids that activate autophagy and promote VSMC de-differentiation, rapamycin appears to uphold or promote the differentiation of VSMCs in the contractile phenotype [162, 163]; however, the effects of rapamycin-based drugs are unlikely to be due to its activation of autophagy. Rather, the

drugs appear to prevent proliferation and migration by inhibiting S6K1 and regulating the expression of key cell cycle proteins [162– 167]. Drugs and newer compounds shown to inhibit VSMC growth, e.g., verapamil [168] and emodin [169], also induce autophagy, which mediates some of their effects. Such drugs are largely outside the scope of this review and are reviewed elsewhere [170]. Nevertheless, it is important to consider how the effects of autophagy induced by drugs may differ from that induced by mitogens such as PDGF and POVPC. Are these truly divergent forms of autophagy? Or, are the different outcomes due to an emergent phenotype derived from the integration of cell signaling and the autophagic program?

While the synthetic or secretory phenotype is most commonly associated with VSMC de-differentiation, autophagy may also be involved in the development of the osteochondrogenic VSMC phenotypes. Vascular calcification occurs with advancing age and is mediated by a switch from a de-differentiated phenotype to a pro-calcificatory phenotype [171]. The differential expression of several genes, including matrix Gla protein, bone morphogenic protein-2, alkaline phosphase, type I collagen and runt-related transcription factor-2, appear to underlie conversion to the calcified VSMC phenotype [171, 172]. Interestingly, calcifying microvesicles have been shown to be a nidus for mineralization in the vessel wall [173], and activation of autophagy appears to prevent their release from VSMCs [72]. While many of the details of how autophagy regulates VSMC calcification are yet unclear, its effects on the actin cytoskeleton and organization of the plasma membrane could be responsible, as other studies have shown a similar regulatory effect of autophagy on exosome release [174]. Whether activation of autophagy could be a viable therapeutic strategy to prevent vascular calcification and ameliorate arterial stiffening remains to be determined.

In macrophages, autophagy appears to be anti-apoptotic, and, in the context of atherosclerosis, predominantly protective. This view is supported by evidence showing that inhibition of autophagy worsens atherosclerosis. Macrophage-specific deletion of Atg5 in LDLR knockout mice results in plaques with increased oxidative stress, apoptosis and necrosis [175]. Furthermore, it was shown in mice that markers of autophagy colocalized with macrophages, but that macrophages had elevated levels of p62, suggesting that autophagy becomes dysfunctional during atherosclerosis. Macrophage-specific deletion of Atg5 resulted in increased plaque formation and proatherogenic inflammasome formation [176]. It is likely that the protective effects of autophagy against atherosclerosis are in part linked with cholesterol metabolism. Lipid droplets in foam cells were shown to be delivered to lysosomes through autophagy, and this process is important for the hydrolysis of cholesterol esters to enable ABCA1-dependent cholesterol efflux [177]. That autophagy could be a therapeutic target was shown by studies in which autophagy was stimulated with mTOR inhibitors, which resulted in selective depletion of macrophages in atherosclerotic plaques [178, 179]. However, stimulation of the autophagic pathway may also be detrimental. Induction of autophagy with toll-like receptor 7 ligands such as imiquimod or treatment with everolimus results in autophagy-independent increases in cytokine production, which could lead to induction of adhesion molecules, infiltration of inflammatory cells, and plaque enlargement [180, 181]. It appears that therapies for atherosclerosis targeting autophagy in macrophages are promising, but that the degree of autophagic activation would need to be tightly modulated to achieve a therapeutic window that reduces macrophage content in lesions.

While much is known regarding the role for autophagy in macrophage foam cell formation, comparatively little is known regarding how it impacts VSMC transition to macrophage-like cells. Cholesterol loading of cultured VSMCs as well as exposure of the cells to oxLDL not only results in autophagy [100, 113], but also in the expression of multiple macrophage

markers [182, 183], which suggests an association between the two processes. Few causative links, however, have been established. Interestingly, while treatment of VSMCs with HNE increases autophagy, it does not appear to result in a switch to a synthetic phenotype (unpublished observations), as is shown with POVPC [155, 156]. Rather, HNE-induced autophagy promotes the accumulation of intracellular lipid droplets and the attainment of a foam cell-like phenotype, which is fully inhibited by 3-MA and partially inhibited by the JNK inhibitor SP600125 (unpublished observations and [184]). How autophagy regulates such macrophage features of VSMCs and why structurally different, autophagy-inducing oxidized lipid products have divergent effects on cell phenotype remain to be elucidated.

SUMMARY AND PERSPECTIVE

Although autophagy was shown to occur in VSMCs of diseased vessels over half a century ago, only now is its importance to VSMC function and plasticity appreciated. Particular reactive species, growth factors, and lesional constituents induce VSMC autophagy, which appears to regulate conversion to the synthetic, osteochondrogenic, and macrophage-like cell phenotypes (Fig. 2). The cell signaling pathways underlying the induction of autophagy by these stimuli involve the usual suspects and processes, such as ER stress, mitogen-activated protein kinases, Akt, and AMPK. However, uncertainty exists regarding how such stimuli, cell signaling pathways, and transcriptional processes integrate in the context of disease to elude different VSMC phenotypes. A current hypothesis with respect to transition to the synthetic phenotype is that autophagy removes contractile elements, which integrates with known transcriptional programs to hasten phenotype transition and the VSMC response to injury.

How does VSMC autophagy regulate vascular diseases such as atherosclerosis, hypertension, and restenosis? This appears to be the fundamental, unanswered question with respect to the importance of VSMC autophagy in disease. Nonetheless, it is becoming apparent that the impact of autophagy in VSMCs in the diseased vasculature might differ largely from that occurring in other cell types such as macrophages. Autophagy in VSMCs, in most but not all cases, promotes cell survival and can be an important means for the cell to remove damaged proteins and detritus as well as to develop into a highly proliferative and migratory phenotype, e.g., [84, 122, 123]. In contrast, the effects of autophagy in macrophages have been shown to have opposite effects, predominantly promoting cell death, e.g. [178, 179]. This disparity in cell responses to autophagy could be of therapeutic benefit in the context of atherosclerosis: promoting vascular autophagy may help stabilize atheromas, while at the same time promote the selective depletion of macrophages. In contrast, promoting autophagy, as least the form shown to occur in response to factors such as PDGF [122] and Shh [123], could accelerate the growth of neointimal lesions and promote the development of restenosis. Further understanding of how autophagy regulates the function of lesional cells will undoubtedly be helpful in understanding whether its modulation could be a viable option for therapy. However, one aspect regarding autophagy in VSMCs is clear. History has taught us that it is a double-edged sword, and it would appear that autophagy retains this title in VSMCs as well.

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HIGHLIGHTS

- In VSMCs, autophagy is activated by stimuli associated with vascular disease.
- Autophagy appears to regulate VSMC dedifferentiation.
- VSMC autophagy induced by reactive species and growth factors promotes cell survival.
- The regulation of autophagy in VSMCs could have important therapeutic implications.

Figure 1. Essential steps in the process of autophagy

Once activated, autophagy proceeds through several stages, with each stage requiring specific regulatory proteins and complexes for autophagic procession. In the initiation or nucleation stage, autophagosomal structures are formed from pre-existing plasma or organellar membranes, which leads to the development of an isolation membrane. This stage requires the activation of Atg1/ULK1 and the class III PI3K (PI3KIII)/Beclin 1 (BCL1) complex. In the elongation and maturation stages, the Atg12–Atg5 and LC3-phosphatidylethanolamine (PE) conjugation systems collaborate to promote conjugation of LC3 to PE and affix LC3 to the autophagosomal membrane. Specifically, the Atg12–Atg5–Atg16L complex participates in the elongation of the autophagic membrane and Atg4

cleaves Pro-LC3 to generate LC3-I, which exposes a PE conjugation site at the COOH-terminal glycine residue. The conjugation of LC3-I to PE then proceeds from the sequential actions of Atg7 and Atg3. The LC3-II formed is then specifically associated with autophagosomes, and the phagosomal structure closes (maturation). After fusion with lysosomes, the cargo sequestered within autophagosomes is degraded by lysosomal acid hydrolases. The hydrolyzed contents, e.g., amino acids and lipids, can then be released for metabolic recycling.

Figure 2. Working model of the regulation of VSMC phenotype by autophagy

During vascular diseases such as atherosclerosis and hypertension, or upon percutaneous interventions, contractile VSMCs are exposed to autophagic stimuli. These stimuli include reactive species, including 4-hydroxynonenal (HNE), nonenal, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), acrolein, 7-ketocholesterol, oxidants such as superoxide and advanced glycation end products (AGEs). In addition, growth factors, cytokines and components of arterial lesions such as platelet-derived growth factor (PDGF)-BB, osteopontin, sonic hedgehog (Shh), tumor necrosis factor- α , oxidized LDL, and inorganic phosphate (Pi) also promote activation of autophagy in VSMCs. Nutrient stress and hypoxia also may stimulate VSMC autophagy. Autophagy induced by some of these factors has been shown to promote the conversion of contractile VSMCs to a synthetic and macrophage-like cell phenotype. In contrast, the osteochondrogenic VSMC phenotype appears to be suppressed by activation of autophagy, which prevents release of matrix vesicles (MVs). Autophagy in VSMCs may be a critical regulator of atherosclerotic plaque

stability, the development of hypertension, VSMC proliferation and migration in restenotic lesions, and vascular calcification.

Table 1Evidence for autophagy occurring in VSMCs *in vivo*.

Species	Disease/Context	Tissue	Method of Detection *	Reference
Human	Atherosclerosis	Aorta and coronary artery	EM	[35]
Rat	Artery ligation	Carotid artery	EM	[151]
Rabbit	Atherosclerosis	Aorta	EM	[36]
Rabbit	Atherosclerosis	Coronary artery	EM	[43]
Baboon	Atherosclerosis	Aorta	EM	[44]
Rat	Anoxia	Arterioles in the pancreas and submandibular gland	EM	[45]
Rat	Hypertension	Thoracic aortic	EM	[46]
Rat	Hypertension	Thoracic aortic	EM	[47]
Rabbit	Atherosclerosis	Aorta	EM	[48]
Monkey	Atherosclerosis	Ascending aortic arch	EM	[49]
Rabbit	Atherosclerosis	Aorta	EM	[50]
Rat	Pulmonary artery hypertension	Pulmonary artery	Western blotting and immunocytochemistry	[51]

* EM, electron microscopy