This Review is in a thematic series on **Autophagy in Health and Disease,** which includes the following articles:

Molecular Mechanisms of Autophagy in the Cardiovascular System
Autophagy in Vascular Disease
The Role of Autophagy in Vascular Biology
Therapeutic Targeting of Autophagy: Potential and Concerns in Treating Cardiovascular Disease
Untangling Autophagy Measurements: All Fluxed Up
Mitochondria and Autophagy

Daniel Klionsky, Guest Editor

Autophagy in Vascular Disease

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Abstract: **Autophagy is a reparative, life-sustaining process by which cytoplasmic components are sequestered in double-membrane vesicles and degraded on fusion with lysosomal compartments. Growing evidence reveals that basal autophagy is an essential in vivo process mediating proper vascular function. Moreover, autophagy is stimulated by many stress-related stimuli in the arterial wall to protect endothelial cells and smooth muscle cells against cell death and the initiation of vascular disease, in particular atherosclerosis. Basal autophagy is atheroprotective during early atherosclerosis but becomes dysfunctional in advanced atherosclerotic plaques. Little is known about autophagy in other vascular disorders, such as aneurysm formation, arterial aging, vascular stiffness, and chronic venous disease, even though autophagy is often impaired. This finding highlights the need for pharmacological interventions with compounds that stimulate the prosurvival effects of autophagy in the vasculature. A large number of animal studies and clinical trials have indicated that oral or stent-based delivery of the autophagy inducer rapamycin or derivatives thereof, collectively known as rapalogs, effectively inhibit the basic mechanisms that control growth and destabilization of atherosclerotic plaques. Other autophagy-inducing drugs, such as spermidine or add-on therapy with widely used antiatherogenic compounds, including statins and metformin, are potentially useful to prevent vascular disease with minimal adverse effects. (*Circ Res*. 2015;116:468-479. DOI: 10.1161/CIRCRESAHA.116.303804.)**

Key Words: atherosclerosis ■ autophagy ■ vascular diseases

Autophagy is a complex intracellular process that delivers cytoplasmic constituents for degradation into lysosomes.[1,2] Three main types of autophagy have been described: (1) microautophagy, comprising direct engulfment of cytoplasmic material by lysosomes via inward invaginations of the lysosomal membrane, (2) macroautophagy, characterized by formation of double-membrane sequestering compartments termed autophagosomes that fuse with lysosomes for delivery of cytoplasmic cargo, and (3) chaperone-mediated autophagy, mediated by a chaperone complex and lysosomal-associated membrane protein type 2A to degrade cytosolic proteins with a specific targeting motif. The term autophagy usually refers to macroautophagy, which is the most prevalent and best-studied form of autophagy. Also in this review, we will focus exclusively on macroautophagy, further cited as autophagy.

Autophagy occurs at basal levels in most tissues to allow constitutive turnover of cytosolic components but is stimulated by environmental stress-related signals (eg, nutrient deprivation and oxidative injury) to recycle nutrients and to generate energy for maintenance of cell viability in unfavorable conditions.[1] In addition to cellular stress, basal autophagy can be intensified by specific drugs,[3] indicating that the autophagic machinery is a potential therapeutic target for diverse diseases. Indeed, given that autophagy is involved in the prevention

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Nonstandard Abbreviations and Acronyms	
AAA	abdominal aortic aneurysm
AMPK	AMP-activated protein kinase
ATG	autophagy-related gene
EC	endothelial cell
ER	endoplasmic reticulum
LC3	microtubule-associated protein 1 light chain 3
mTOR	mammalian target of rapamycin
NO	nitric oxide
oxLDL	oxidized low-density lipoprotein
PAH	pulmonary arterial hypertension
ROS	reactive oxygen species
SMC	smooth muscle cell

of different human pathological conditions, including heart and liver disease, cancer, neurodegeneration, as well as infectious and metabolic disorders,[4–6] the development of highly specific autophagy modulators has become a major clinical priority. Along these lines, it is noteworthy that autophagy plays an essential role in embryogenesis,[7] aging,[8] regulation of blood glucose and amino acids,[9] thrombosis,[10] and lipid metabolism.[11] Systemic or tissue-specific deletion of autophagy-related (*Atg*) genes in various organisms, including mice, may lead to serious malformations and even death,[12] supporting the general hypothesis that autophagy is an essential process that contributes to health and overall well-being.

Despite the protective actions of autophagy in human physiology and disease, the role of autophagy in the vasculature is poorly understood, probably because of the multifarious actions of the autophagic process.[13] Undoubtedly, overactivation of autophagy is not always protective but may cause cell death with unique morphological features distinct from apoptosis and necrosis, sometimes referred to as autosis or autophagic cell death.[14,15] The latter term is controversial because evidence linking autophagy to cell death is often lacking or not convincing.[16] Another factor that complicates the interpretation of autophagy data is that autophagy can be selective or nonselective. Selective autophagy specifically targets damaged or superfluous organelles (eg, mitochondria degraded by a selective form of autophagy termed mitophagy) using a specific set of receptors and autophagic scaffold proteins, whereas nonselective autophagy is important for bulk turnover of cytosolic material.[2] Also noteworthy is that detection of autophagosomes via transmission electron microscopy, currently the gold standard for monitoring autophagy, as well as staining of autophagic proteins in the vessel wall by standard immunohistochemistry is challenging and often leads to misinterpretation.[17,18] Still, recent reports from our group and others have demonstrated that impaired autophagy has a major effect on the functionality of the vessel wall and the initiation of vascular disease. In this review, we first summarize recently derived insights about the role of autophagy in normal vascular function. Next, we describe the current knowledge of autophagy in vascular disease and how the autophagic machinery can be modulated with pharmacological compounds to treat unstable atherosclerotic plaques and other vascular disorders.

Autophagy in the Normal Vessel Wall

Autophagy Is a Cytoprotective Mechanism

Autophagy in vascular endothelial cells (ECs) is a protective mechanism against many pathophysiological stimuli, including oxidized low-density lipoprotein (oxLDL),[19] reactive oxygen species (ROS),[20] lipopolysaccharides,[21] hypoxia,[22] and advanced glycation end products.[23] Moreover, several natural compounds with anti-inflammatory or antioxidant activity (eg, curcumin, vitamin D, resveratrol, or the analogue pterostilbene)[24–28] promote autophagy in ECs to protect against endothelial inflammation or oxidative stress. However, oxidative stress induced by ROS or oxLDL may also affect the endoplasmic reticulum (ER),[20,29] inducing deregulation of cytosolic calcium, a condition known to trigger ER stress and apoptosis. Splicing of the mRNA of X-box–binding protein 1, which is an important event during ER stress, allows formation of a potent transcription factor in ECs that triggers an autophagic response through transcriptional activation of the autophagy regulator Beclin 1.[30] Because silencing of Beclin 1 in ECs reduces the proportion of annexin V staining but not oxLDL-induced apoptosis, activation of endothelial autophagy by oxLDL seems to occur independent of ER stress and downstream apoptotic signaling. Instead, the autophagic program in oxLDL-exposed ECs is considered to be involved in the externalization of phosphatidylserine as an eat-me-signal for optimal clearance of dead cells.[29]

Analogous with ECs, many vascular disease-related stimuli, such as ROS and oxidized lipids, have been shown to activate autophagy in vascular smooth muscle cells (SMCs) as a prosurvival mechanism. An overload of free cholesterol, for example, triggers autophagy in SMCs as illustrated by increased formation of autophagic vacuoles and conversion of microtubule-associated protein 1 light chain 3 (LC3)-I into the autophagosome-specific LC3-II and promotes SMC survival, possibly by improving the clearance of damaged organelles.[31] Inhibition of autophagy by treatment with 3-methyladenine leads to increased ER stress and mitochondrial depolarization, whereas rapamycin treatment reduces ER stress by stimulating autophagy. Autophagy in SMCs may also be activated by cytokines and growth factors. The cytokine osteopontin has been shown to activate autophagy in SMCs as shown by autophagosome formation and increased LC3-II/LC3-I ratio.[32] Furthermore, tumor necrosis factor-α and platelet-derived growth factor induce autophagy and protect SMCs against cell death.[33,34]

All together, we may conclude that autophagy serves as an important survival pathway in ECs and SMCs, even though its protective effects could ultimately be overwhelmed by proapoptotic mechanisms. Given that autophagy acts as a prosurvival mechanism, it is plausible to assume that defects in the autophagic machinery could aggravate cell death. Surprisingly, autophagy defective SMCs elicited by genetic deletion of the essential autophagy gene *Atg7* are more resistant to oxidative stress-induced cell death when compared with controls (M. Grootaert, unpublished data, 2014). This effect is attributed to nuclear translocation of the nuclear factor erythroid 2–related factor 2 resulting in upregulation of several antioxidative enzymes, such as glutathione S-transferase

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α and NAD(P)H:quinone oxidoreductase 1. Thus, the nuclear factor erythroid 2–related factor 2 pathway is activated in SMCs as a protective backup mechanism to maintain cell survival in case of defective autophagy.

Autophagy Regulates SMC Phenotype and Proliferation

Besides its role in cell survival, autophagy may also regulate SMC phenotype and proliferation. Treatment of SMCs with platelet-derived growth factor induces autophagy via an AMP-activated protein kinase (AMPK)–independent and mammalian target of rapamycin (mTOR)–independent mechanism and results in decreased expression of contractile proteins, whereas synthetic SMC markers are upregulated.[34] Moreover, platelet-derived growth factor–induced autophagy is associated with an enhanced potential to migrate and to proliferate, thereby promoting a synthetic SMC phenotype. Conversely, inhibition of autophagy by 3-methyladenine or spautin-1 stabilizes the contractile phenotype and reduces platelet-derived growth factor–induced proliferation. These findings are in line with the current hypothesis that transition to a synthetic SMC phenotype is associated with the removal of contractile elements by autophagy.[35] Other evidence that uncovers a link between autophagy and SMC proliferation points to the secreted protein sonic hedgehog. This protein induces SMC proliferation by activation of autophagy in an AKT-dependent manner.[36] It plays a critical role in the pathogenesis of vascular disease and is particularly involved in the regulation of SMC growth, vasculogenesis, and angiogenesis. Treatment with 3-methyladenine, however, inhibits SMC proliferation caused by sonic hedgehog overexpression.

Interestingly, recent evidence in our laboratory showed a novel link among autophagy, SMC proliferation, and phenotype. Defective autophagy in SMCs, elicited by genetic deletion of the essential autophagy gene *Atg7*, leads to the induction of stress-induced premature senescence as shown by cellular hypertrophy, p16-mediated G1-proliferative arrest, increased migration and augmented collagen synthesis (M. Grootaert, unpublished data, 2014). This new data contribute to our knowledge of how autophagy regulates SMC growth.

Autophagy Preserves Endothelial Function

Current knowledge of EC autophagy is mainly based on in vitro experiments using either human umbilical vein ECs or bovine aortic ECs. Indeed, except for ECs covering advanced atherosclerotic plaques,[37] thorough in vivo evidence for EC autophagy is lacking. Notwithstanding, growing evidence reveals that autophagy is an essential in vivo process mediating accurate EC function. First, autophagy regulates maturation and secretion of von Willebrand factor.[10] As a consequence, endothelial-specific deletion of the essential autophagy gene *Atg7* in mice results in impaired epinephrine-stimulated von Willebrand factor release, reduced levels of high-molecular weight von Willebrand factor multimers, and significantly increased bleeding times.[10] Second, impaired autophagy contributes to arterial aging and represents a potential cause of age-related arterial dysfunction.[38] In both humans and mice, aging in ECs is associated with decreased expression levels of several autophagy marker proteins (eg, Beclin 1, LC3-II), a reduction in nitric oxide (NO) bioavailability and arterial endothelium-dependent dilatation, as well as increased levels of oxidative stress and inflammation.[38] Treatment of old mice with the autophagy enhancer trehalose or spermidine could rescue this phenotype. Recent evidence indicates that miR-216a as a microRNA is upregulated during endothelial aging to repress expression of 2 autophagy-related genes, Beclin 1 and Atg5.[39] miR-216a also induces oxLDL accumulation and monocyte adhesion in ECs, whereas downregulation of miR-216a exerts a protective antiatherogenic role against oxLDL treatment.[39] These findings indicate that miR-216a is an important factor between endothelial dysfunction and autophagy and may have a relevant role in aging-related vascular disorders. Third, autophagy contributes to the upregulation of endothelial NO synthase expression, at least under steady laminar shear stress, and inhibits endothelin-1 expression, a specific protein marker of endothelial dysfunction and natural counterpart of the vasodilator NO. When pretreated with 3-methyladenine, endothelial NO synthase expression in ECs is inhibited and endothelin-1 expression is restored.[40] Overall, we may conclude that autophagy is a key cellular process in ECs that preserves endothelial function and prevents cardiovascular disease.

Autophagy in Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial wall and the leading cause of death in the developing countries.[41,42] Risk factors of atherosclerosis include not only hypertension, hypercholesterolemia, diabetes mellitus, obesity, and smoking[43] but also aging is considered an important contributing factor of this widespread vascular disease.[44] Atherosclerosis is characterized by the formation of plaques in large- and medium-sized arteries comprising SMCs, inflammatory cells (such as macrophages, dendritic cells, T lymphocytes, and mast cells), extracellular matrix, and lipids.[41,42] Rupture of atherosclerotic plaques is facilitated by thinning of the fibrous cap, caused by SMC death and extracellular matrix degradation, and may lead to clinical complications, such as myocardial infarction, stroke, and sudden death.[41,42] Changes in lifestyle (diet and exercise), the use of cholesterol-lowering drugs (statins), and the application of balloon angioplasty or stenting have led to reduced patient morbidity and mortality.[45] However, to develop new and improved therapeutic strategies, a better understanding of the underlying mechanism of plaque progression and rupture is necessary. A recent body of evidence suggests that autophagy plays a major role in modulating atherogenesis and atherosclerotic plaque stability and may provide new opportunities for the treatment of atherosclerosis.[46–48]

Autophagy Is Stimulated During Plaque Formation

Recent transmission electron microscopy analysis suggests that autophagy occurs in all major cell types of human atherosclerotic plaques (ie, ECs, macrophages, and SMCs), present in the fibrous cap and around the necrotic core.[37] The autophagic cells are characterized by engulfment of amorphous material in membranous enclosures and cytoplasmic vacuoles, distinguishable from lipid droplets and lysosomes, and are found at relatively low frequencies (≈1.5% for each cell type)

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comparable with the incidence of apoptosis.[37] These findings are supported by Western blot analysis of lysates from advanced human plaques showing elevated levels of LC3-II.[49] Of note, LC3-II is not detectable in nonatherosclerotic arteries, indicating that autophagy is strongly induced during atherogenesis.

Autophagy Has Diverse Effects in Atherosclerotic Plaques

During the past few years, the role of autophagy in the pathogenesis of atherosclerosis has been thoroughly investigated by several research groups. Autophagy is involved in macrophage reverse cholesterol transport and regulates the delivery of lipid droplets to lysosomes in macrophage foam cells, where lysosomal acid lipase-dependent lipolysis leads to the generation of free cholesterol for efflux.[50] Lipid-loaded macrophages lacking the essential autophagy gene *Atg5* showed reduced cholesterol efflux. Upregulation of mTOR and p-mTOR protein is observed in macrophage-derived foam cells, whereas blocking mTOR expression with specific siRNA suppresses foam cell formation.[51] Thus, autophagy promotes cholesterol efflux from macrophage foam cells, thereby contributing to the regression of atherosclerotic plaques.[52] Along these lines, PPM1D phosphatase deficiency may prevent foam cell formation and ultimately plaque development by activation of autophagy-dependent cholesterol efflux via an ATM-dependent inhibition of mTOR.[53]

Two articles provided new insights in the role of macrophage autophagy in atherosclerotic plaque development. First, Liao et al[54] reported a protective role of macrophage autophagy in advanced atherosclerosis. They showed that macrophage-specific deletion of *Atg5* in LDLR$^{-/-}$ mice exacerbates atherosclerotic plaque development after 12 and 16 weeks of Western-type diet by increasing macrophage apoptosis and necrosis.[54] Macrophage apoptosis is augmented in *Atg5*-deficient macrophages by increased NADPH-oxidase activity resulting in increased ROS generation.[54] Besides the increase in oxidative stress, defective macrophage autophagy results in reduced phagocytic clearance of apoptotic macrophages, promoting plaque necrosis. Second, defective autophagy in macrophages is associated with hyperactivation of the inflammasome, stimulating atherosclerotic plaque progression.[55] The hyperactivation of the inflammasome NLRP3 is mediated by the accumulation of cholesterol crystals resulting in a proatherogenic caspase-1–mediated interleukin-1β response.[55] In addition, LOX1-mediated autophagy and mitochondrial DNA damage play an essential role in NLRP3 inflammasome activation in macrophages.[56] Inhibition of ROS and induction of autophagy decreases NLRP3 expression, whereas autophagy inhibition exerts the opposite effect.

Recent work in our laboratory has demonstrated an important role of autophagy in the regulation of SMC survival and phenotype with major implications for atherosclerosis (M. Grootaert et al, unpublished data, 2014). As mentioned above, autophagy-deficient SMCs show upregulation of several nuclear factor erythroid 2–related factor 2–dependent antioxidative enzymes, which is not observed in autophagy-deficient macrophages. These results not only support previous findings of Liao et al[54] but also indicate a cell-type–dependent role of autophagy in the regulation of cell survival. Moreover, SMC-specific deletion of *Atg7* in ApoE$^{-/-}$ mice accelerates atherosclerotic plaque development after 10 weeks of Western-type diet. Besides the increase in plaque size, the atherosclerotic lesions in SMC-specific *Atg7* knockout mice were also more advanced as shown by increased plaque cell death, plaque macrophages, fibrous cap thickness, and collagen content. The accelerated atherogenesis is associated with the development of SMC senescence as shown by nuclear hypertrophy and p16 upregulation in plaque SMCs. After 14 weeks of Western-type diet, lesions were still characterized by an increase in fibrous cap thickness and collagen content but showed no differences in plaque size and plaque cell death, indicating that defective autophagy in SMCs accelerates atherogenesis without worsening plaque stability. The latter is not in line with the findings in the macrophage-specific *Atg5* knockout mice, in which atherosclerotic plaques show severe destabilization after 16 weeks of Western-type diet. It is also important to note that autophagy-deficient macrophages do not develop senescence (M. Grootaert, unpublished data, 2014). We may conclude that defective autophagy has diverse effects on macrophages and SMCs, yet it exerts in both cases detrimental effects on atherosclerotic plaque development. Therefore, inhibition of autophagy would be unfavorable as therapeutic approach in the treatment of atherosclerosis.

Autophagy in Early Versus Advanced Atherosclerosis

According to Razani et al,[55] autophagy becomes dysfunctional when the atherosclerotic plaque develops. In this study, ApoE$^{-/-}$ mice were fed a Western-type diet, and aortic levels of the autophagy substrate SQSTM1/p62 were evaluated as an indicator for defective autophagy. The expression of the SQSTM1/p62 protein was dramatically increased in the atherosclerotic aortas and was further elevated with increasing age and plaque burden.[55] It remains unclear how a prosurvival pathway, such as autophagy, becomes dysfunctional in atherosclerotic plaques, although some theories can be mentioned. The general concept is that autophagy is activated in early lesions to protect plaque cells against oxidative injury, metabolic stress, and inflammation (Figure 1). Indeed, in case of mild oxidative stress, autophagy contributes to cellular recovery by degrading damaged proteins and intracellular material.[57] During severe oxidative stress, however, autophagy becomes insufficient to remove damaged mitochondria so that leakage of cytochrome *c* from damaged mitochondria and activation of the intrinsic apoptotic pathway may occur (Figure 2). Also oxidative damage of the lysosomal membrane may lead to the release of lysosomal hydrolases, which causes damage of cytosolic proteins and organelles and induces apoptosis.[57] Moreover, autophagy in combination with severe oxidative stress leads to the formation of ceroid, an insoluble complex of proteins associated with oxidized lipids, present in human advanced atherosclerotic lesions.[58] Ceroid accumulates in lysosomes to which enzymes are distributed in a useless attempt to facilitate their degradation.[59] As a result, the lysosomal hydrolases can no longer be involved in active autolysosomes, promoting autophagy impairment and apoptosis induction. Failure of the autophagic process stimulates further

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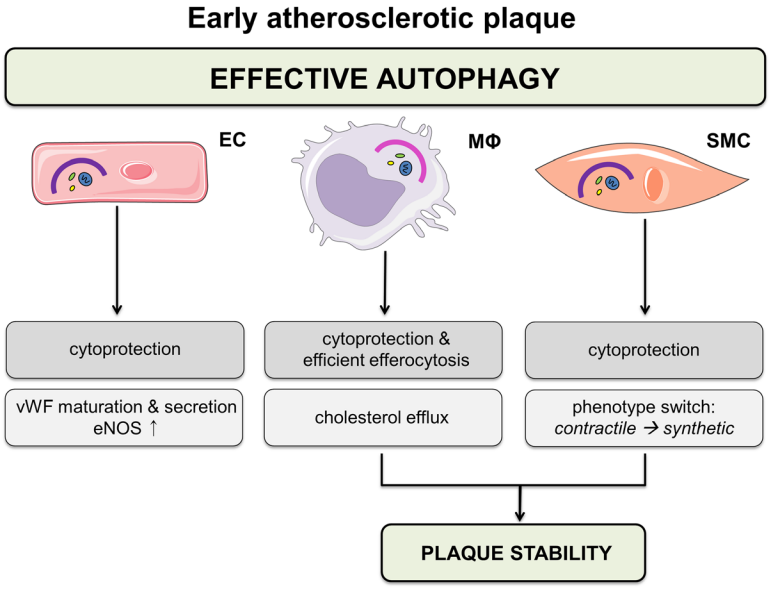

Figure 1. Role of autophagy in early atherosclerosis. In early atherosclerotic plaques, autophagy is activated to protect plaque cells against oxidative injury (reactive oxygen species, oxidized low-density lipoprotein), metabolic stress, and inflammation (cytokines). In this way, autophagy contributes to cytoprotection and prevents apoptosis. In endothelial cells (EC), autophagy mediates maturation and secretion of von Willebrand factor (vWF), as well as upregulation of endothelial nitric oxide synthase expression (eNOS). Moreover, autophagy promotes efficient clearance of dead cells by macrophages (MΦ), as well as cholesterol efflux from foam cells. In addition, it induces a phenotypic switch of smooth muscle cells (SMC). Overall, autophagy preserves normal cellular function in early atherosclerotic plaques to maintain plaque stability.

accumulation of damaged mitochondria, increased ROS generation, and enhanced formation of ceroid-containing lysosomes (Figure 2).[59] Hence, the cross talk between autophagy and apoptosis plays an essential role in modulating atherosclerotic plaque progression and stability.

The large amount of NO produced by plaque macrophages may be considered as another important underlying mechanism involved in local autophagy impairment. Macrophages present in human atherosclerotic plaques express high levels of the inducible isoform of NO synthase, which produces large amounts of NO. The latter can react with superoxide to form peroxynitrite, a potent oxidant and nitrating agent that causes tissue damage by oxidation of lipids and nitrosylation of proteins.[60] Recent evidence addresses a role of NO in inhibition of autophagy (Figure 2).[61]

Given that the accumulation of SQSTM1/p62 protein in atherosclerotic aortas is further elevated with increasing age,[55] aging may be a crucial factor in linking autophagy impairment with atherosclerosis. Multiple reports indicate that autophagic activity declines with aging as shown by the reduced expression of autophagy-related proteins in aged tissue.[62] The reason for the age-dependent reduction in autophagy involves a defect in the clearance of autophagic vacuoles because of failure of lysosomal hydrolases.[63] Because atherosclerosis is classified as an age-related disease and aging is recognized as an important risk factor,[44] it is conceivable that aging contributes to impaired autophagy and thereby aggravates atherosclerosis (Figure 2).

Autophagy in Abdominal Aortic Aneurysm

Abdominal aortic aneurysm (AAA) is a common vascular disease characterized by an abnormal enlargement or bulging of the aortic wall. Such dilation and structural weakening of the abdominal aorta may eventually lead to rupture of the vessel wall, yielding about 15 000 deaths in the United States every year. Although the molecular mechanisms underlying

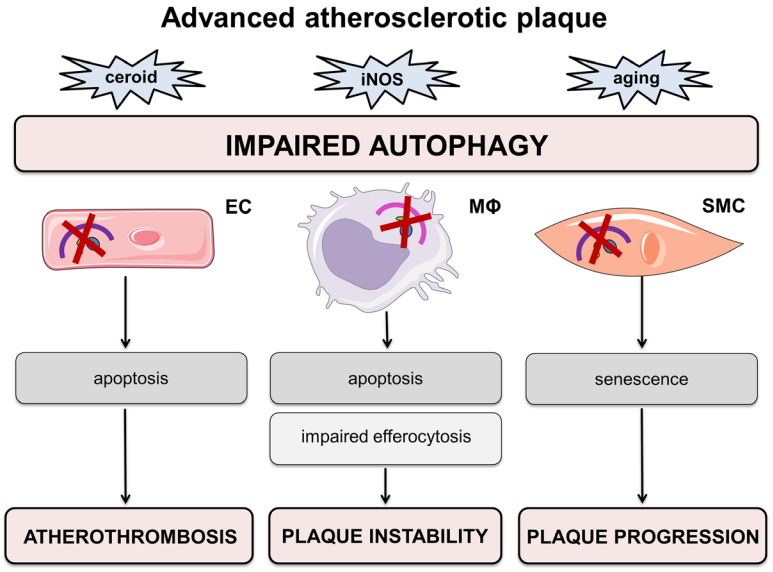

Figure 2. Role of autophagy in advanced atherosclerosis. In advanced plaques, ceroid deposition, high amounts of nitric oxide (NO) via iNOS expression, and aging impair autophagy. Failure of the autophagic process further stimulates accumulation of damaged organelles, increases reactive oxygen species generation and enhances formation of ceroid-containing lysosomes. Impaired autophagy in endothelial cells (EC) is associated with endothelial dysfunction and apoptotic cell death, possibly leading to atherothrombosis. In macrophages (MΦ), impaired autophagy results in increased sensitivity to apoptotic stimuli and impaired efferocytosis, resulting in plaque instability. Smooth muscle cells (SMC) undergo senescence, which accelerates plaque progression.

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AAA remain obscure, inflammation seems to play a key role in the pathogenesis of the disease. Inflammatory cells may infiltrate AAA and produce matrix-degrading enzymes, ROS, and several proinflammatory cytokines, such as tumor necrosis factor-α, interleukin-6, and osteopontin, further triggering inflammation and SMC death.[64,65] In line with these findings, microarray analysis of AAA revealed upregulation of several autophagy-related genes, including *LC3*, *ATG4B*, *BECLIN 1*, *BNIP3*, and *VPS34*.[32] The LC3-II to LC3-I protein ratio is also increased, suggesting induction of autophagy. Because osteopontin is the highest expressed gene in AAA (125-fold induction versus normal aorta),[32] its role in autophagy and SMC death was recently investigated in more detail. Using cultured SMCs, it was demonstrated that osteopontin significantly increases the formation of autophagosomes and the expression of autophagy-related genes through activation of the receptors integrin/CD44 and enhanced p38 MAPK signaling.[32] Further evidence indicates that osteopontin-induced autophagy does not prevent but contributes to SMC death.[32] Indeed, pharmacological stimulation of autophagy by rapamycin exacerbates osteopontin-induced SMC death, whereas inhibition of autophagy by 3-methyladenine brings the opposite. It is likely that depletion of SMCs by autophagy-mediated death contributes to a reduction of cellularity and to impaired repair and maintenance of the extracellular matrix in AAA. In view of this finding, autophagy inhibition could be a potential target in the treatment of AAA disease. Similar observations have been previously reported for apoptosis, which is an another type of SMC death that frequently occurs in AAA and promotes AAA rupture.[66] Yet, recent evidence indicates that rapamycin is remarkably effective in preventing the progression of established aneurysms.[67] Probably, rapamycin may limit AAA through anti-inflammatory effects, analogous with its outcome in atherosclerosis (vide infra), but not via induction of autophagy.

Autophagy in Arterial Aging

In contrast to AAA where autophagy could be stimulated because of the marked upregulation of osteopontin, accumulating evidence indicates that vascular aging is associated with impaired autophagy and may contribute to age-related endothelial dysfunction and arterial stiffness.[38,68] Supplementation of the autophagy inducer spermidine reverses age-associated stiffening of large elastic arteries,[68] a condition that may significantly improve the morbidity and mortality of patients with cardiac disease as aortic stiffness, and specifically aortic pulse wave velocity, has been increasingly recognized as an accurate predictor of cardiovascular risk.[69]

Autophagy in Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a complex and progressive disease characterized by elevations in pulmonary arterial pressure and subsequent right ventricular failure. The underlying mechanism is unclear but involves excessive proliferation and apoptosis resistance in pulmonary SMCs, as well as inflammation and endothelial dysfunction. On the basis of recent reports, we found that autophagy seems to have a dual role in the pathogenesis of PAH. Autophagy is markedly increased in pulmonary vascular cells from patients with PAH, which is thought to be a protective mechanism because LC3B knockout mice display enhanced PAH in response to chronic hypoxia.[70] Moreover, the sex hormone 17β-estradiol (E2) that has protective properties in PAH increases lung LC3-II expression in chronically hypoxic rats.[71,72] However, in contrast to the findings above, also inhibition of autophagy via chloroquine treatment exerts beneficial effects albeit in a different, monocrotaline-induced model of PAH.[73] Chloroquine inhibits the lysosomal degradation of bone morphogenetic protein type II receptor, a protein required in pulmonary arteries for inhibition of SMC proliferation and increased apoptosis through Smad signaling.

Autophagy in Other Vascular Diseases

Little is known about the autophagy in other vascular disorders, such as chronic venous disease. However, given that autophagy-deficient SMCs are hypertrophic (vide supra) and varicose veins develop SMC hypertrophy,[74] it is tempting to speculate that autophagy in SMCs of varicose veins might be impaired. Another observation that points to impaired autophagy in vascular disease, even though thorough evidence is lacking, is the accumulation of cytosolic ubiquitin inclusions in calcified, degenerative aortic valves.[75] In the early 2000s, granular cytoplasmic ubiquitin inclusions were considered to be an attractive marker for autophagic degeneration of cardiomyocytes during heart failure.[76] However, more recent evidence indicates that these inclusions may result from defective autophagy as ubiquitin-positive cytoplasmic inclusions colocalize with enhanced levels of SQSTM1/p62,[18] a selective substrate of autophagy that accumulates in cells when autophagy is inhibited.

Pharmacological Induction of Autophagy in Vascular Disease

Stent-Based Delivery of Rapamycin and Rapalogs

Rapamycin, also known as sirolimus, is naturally produced by the bacterium *Streptomyces hygroscopicus* and was initially used as an antifungal agent. Later, however, it was found to have potent immunosuppressive effects in mammals so that it could be used in autoimmune disorders and after organ transplantation.[77,78] Because rapamycin is a potent inhibitor of mTOR complex 1 (mTORC1), rapamycin is also useful as a proliferation inhibitor to prevent SMC migration and restenosis after angioplasty.[79] Interestingly, rapamycin-mediated mTORC1 inhibition mimics nutrient deprivation and leads to a robust induction of autophagy. As a consequence, rapamycin is currently the most frequently used pharmacological agent to induce autophagy both in vitro and in vivo. Derivatives of rapamycin, collectively known as rapalogs, have been synthesized to improve the pharmacokinetic properties of the parent compound and to reduce its toxicity. These derivatives include everolimus (RAD-001), temsirolimus (CCI-779), zaterolimus (ABT-578), ridaforolimus (AP-23573), and biolimus.[78,80,81] The introduction of drug-eluting stents coated with rapamycin (or a derivative thereof) has started a revolution in the field of interventional cardiology.[82] These stents were superior to any bare metal stent available.[83] Stents releasing

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other cell proliferation inhibitors (eg, paclitaxel), however, proved inferior to rapamycin even though both drugs inhibit SMC proliferation,[84,85] indicating that mechanisms other than just proliferation arrest are involved. Research in our laboratory showed that stent-based delivery of everolimus in rabbit atherosclerotic plaques results in a selective clearance of macrophages without any influence on SMC content.[86,87] Within these plaques, macrophages showed strong vacuolization and underwent cell death associated with autophagy while no such observations were made in SMCs. This finding is strengthened by 2 recent reports showing that inhibition of mTOR signaling with siRNA induces autophagy in macrophages of rabbits or ApoE$^{-/-}$ mice and reduces both plaque burden and macrophage content within the atherosclerotic lesions.[88,89] Everolimus-induced macrophage death most likely occurs because of protein synthesis arrest after mTOR inhibition. Indeed, being a highly active cell type, macrophages are much more sensitive to protein synthesis arrest when compared with SMCs and rapidly initiate cell death,[47] whereas SMCs transform into a quiescent, more contractile phenotype.[90] These findings were confirmed with cycloheximide, a general protein synthesis inhibitor triggering selective macrophage clearance in rabbit plaques.[91] In addition, Hsu et al[92] provided evidence from cultured human THP1 macrophage-derived foam cells that everolimus may potentially inhibit atheroma progression or promote atheroma stabilization through diminished viability of foam cells, decreased matrix degradation, and reduced proinflammatory cytokine secretion. However, it is currently unknown to which degree atheroprotection by rapamycin/rapalogs is specific for macrophage autophagy and the mTORC1-dependent versus mTORC2-dependent signaling pathways. Studying rapalog-treated atherosclerotic mice with a deficiency in macrophage autophagy (eg, ATG5 deficient), mTORC1 (eg, Raptor deficient), or mTORC2 (eg, Rictor deficient) will provide further insights in the specificity and the regulation of macrophage autophagy in atherosclerotic plaques.[93]

Everolimus eluting fully biodegradable vascular scaffolds are now being used in clinical trials with extraordinary results. Biodegradable vascular scaffold are completely bioresorbed in 2 years, thus eliminating any risk for in stent thrombosis and aiding the restoration of vasomotoric functions in the stented segments.[94] Unlike everolimus-coated metallic stents, everolimus-coated biodegradable vascular scaffold trigger a healing process in the vessel wall resulting in lumen enlargement (≤10 mm^2 at 24-month follow-up) and regression of both plaque and media (≤13%).[95] Hitherto, this so-called atheroregression is poorly understood. It could be the result of a volumetric reduction after biodegradation of the scaffold.[95] However, given that everolimus clears macrophages in experimental plaques and stimulates cholesterol efflux via autophagy induction (vide supra), it is conceivable that autophagy is involved although thorough in vivo evidence for this theory is lacking.

Systemic Administration of Rapamycin and Rapalogs

Because atherosclerosis is a systemic disease, patients would likely benefit more from a systemic treatment. Administration of rapalogs in mouse or rabbit models of atherosclerosis, either orally or subcutaneously, results in a marked reduction of both plaque size and plaque complexity despite severe hypercholesterolemia.[96–105] Indeed, rapalogs can prevent accumulation of macrophages and lipids and reduce cell proliferation and intraplaque angiogenesis via mTOR inhibition.[48] However, it is currently not known whether autophagy is involved in these plaque-stabilizing effects. In vitro experiments showed that only high concentrations (μmol/L range), as reached locally with rapalog eluting stents, stimulate autophagy-mediated macrophage death.[86,87] When considering systemic administration, these concentrations are supratherapeutic and unlikely to be achieved in the plasma of any patient. Low concentrations (nmol/L range) of rapalogs can inhibit mTOR, but it is unclear whether they sufficiently induce autophagy in atherosclerotic plaques. Importantly, even low doses of rapalogs are not free of adverse effects and some of them, such as hypercholesterolemia and hyperglycemia, are known triggers of atherosclerosis.[78,106] Hyperlipidemia is frequently observed in rapamycin/rapalog-treated patients and is presumably related

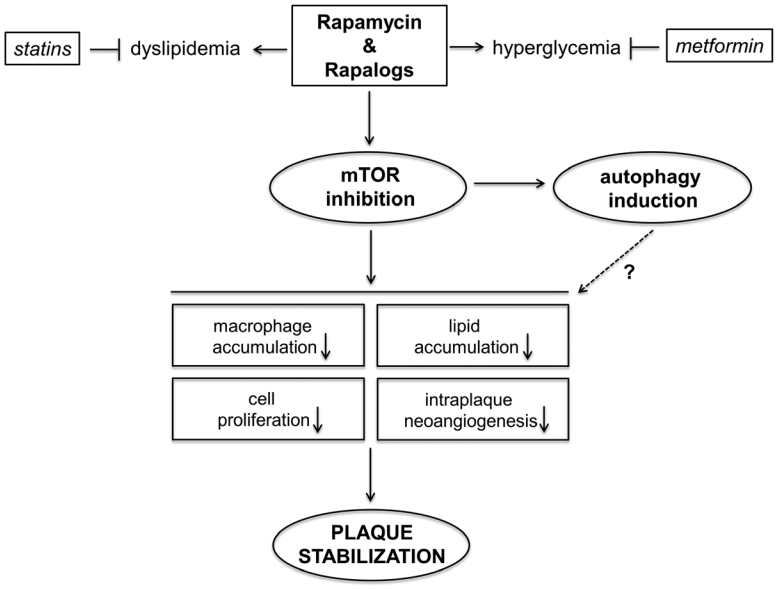

Figure 3. Pharmacological mammalian target of rapamycin (mTOR) inhibition is beneficial for plaque stabilization. Rapamycin/rapalogs are potent mTOR inhibitors and inducers of autophagy and are currently investigated as plaque-stabilizing agents. Several animal and clinical studies using these drugs have demonstrated that mTOR inhibition attenuates smooth muscle cell proliferation, promotes clearance of macrophages and cholesterol from the plaque and might inhibit intraplaque neoangiogenesis, resulting in plaque stabilization. However, it is currently unclear whether these effects are autophagy dependent. Because it is known that these compounds can induce hyperlipidemia and hyperglycemia, we propose combination therapy with a statin and metformin not only to reduce adverse effects but also to stimulate autophagy further.

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Table. Selected Compounds With Autophagy-Inducing Potential

Compound	Mechanism of Autophagy Induction	Effects in Vascular Disease	References
Acid sphingomyelinase	Regulates autophagic flux	Inhibits atherosclerotic plaque formation	112, 113
Calcium channel blockers	Lowers intracytosolic Ca^{2+} levels	Decreases neointima formation through inhibition of SMC proliferation and migration	114, 115
Metformin	Stimulates AMPK	Decreases neointima formation through inhibition of SMC proliferation, migration and inflammation	116
Rapamycin (and rapalogs)	Inhibition of mTORC1	Inhibits atherogenesis	48
		Reduces size and complexity of atherosclerotic plaques	48
		Decreases monocyte migration and SMC proliferation in vitro	105, 117
		Reduces proinflammatory cell mediators in plasma	104
		Prevents lipid accumulation because of downregulation of LDL or scavenger receptors and stimulation of cholesterol efflux	118, 119
		Prevents upregulation of MCP-1	100
		Inhibits neoangiogenesis by reducing accumulation of HIF1-α protein and proliferation of ECs	48, 120
		Clears macrophages from atherosclerotic plaques when released by stents	86, 87
		Induces atheroregression when released by biodegradable vascular scaffolds	95
Resveratrol	Activates SIRT1 and AMPK	Attenuates endothelial inflammation in vitro	27
		Induces SMC differentiation in vitro	121
Spermidine	Cytoplasmic deacetylation nucleic acetylation	Reverses arterial aging	38, 68
Trehalose	Unknown	Reverses arterial aging	38
Valproic acid	Lowers IP_3 levels	Reduces phosphate-induced calcification in aortic ring explants	122

AMPK indicates AMP-activated protein kinase; EC, endothelial cell; HIF1-α, hypoxia-inducible factor; LDL, low-density lipoprotein; MCP-1, monocyte chemotactic protein-1; mTORC1, mammalian target of rapamycin complex 1; SIRT1, sirtuin 1; and SMC, smooth muscle cell.

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to decreased LDLR expression. Rapamycin/rapalogs downregulate hepatic LDLR expression in mice via mTORC1, leading to elevated LDL-cholesterol levels.[107] This finding, together with a recent study, showing that scavenger receptor class B type I expression is decreased in vitro, and that rapamycin induces endothelial dysfunction,[108] may contribute to atherogenesis in rapamycin-treated patients. Although the in vivo contribution of endothelial scavenger receptor class B type I to antiatherogenic processes is still unknown, the net effect of rapamycin/rapalogs on atherosclerosis in humans may not be predictable. Therefore, although rapalogs are getting more attention for the treatment of unstable atherosclerotic plaques,[48,109] combined therapy with other drugs, such as statins, proprotein convertase subtilisin/kexin type 9 inhibitors, or metformin, has been proposed to prevent both atherosclerosis and rapalog-mediated side effects (Figure 3).[48,107]

Other Autophagy Inducers

Several other drugs may act as inducers of autophagy (Table). It should be noted, however, that the majority of these compounds have been tested only in vitro or in disease areas not directly related to the vessel wall (eg, cancer, infectious diseases, and neurodegeneration) so that the effect on vascular disease remains unclear. Yet, the few in vivo studies that have recently been performed in a vascular context offer promising results. Spermidine, for example, is a well-known autophagy inducer that has beneficial effects on the arterial wall. Treatment of aged mice (27–29 months) with spermidine leads to a reduction in oxidative stress, which in turn counteracts aging effects, such as arterial stiffness,[68] frequently observed in old mice. This effect is associated with autophagy induction because expression of autophagy-specific marker proteins is normalized after spermidine treatment.[68] Moreover, recent evidence in our laboratory showed that spermidine prevents necrotic core formation and lipid accumulation in atherosclerotic plaques of $ApoE^{-/-}$ mice most likely through autophagy-dependent stimulation of cholesterol efflux although without changing the size of the plaques (C. Michiels, unpublished data, 2014). However, because L-arginine is a shared substrate in the synthesis of both polyamines and NO, spermidine supplementation might shift L-arginine preferably to NO synthase. This may trigger NO production, as demonstrated by improved acetylcholine-mediated aortic relaxation,[68] and at the same time may inhibit autophagy,[61] at least from a theoretical perspective. Treatment of old mice with the autophagy-enhancing dissacharide trehalose also results in normalization of autophagy marker proteins and improved endothelium-mediated relaxation by NO,[38] supporting the assumption that the reversing effect of spermidine on arterial aging is autophagy related. Finally, it worthwhile to mention that statins, such as simvastatin and atorvastatin, have pleiotropic effects, including the induction of autophagy, that are well beyond their lipid-lowering properties.[110,111]

For other autophagy-inducing drugs, a link between autophagy induction and the prevention (or amelioration) of vascular disease is less obvious. For example, resveratrol is a polyphenol present in red wine that attenuates endothelial inflammation by inducing autophagy in an mTOR-independent

way via several key molecules, including cAMP, AMPK, and sirtuin 1.[27] Moreover, resveratrol induces SMC differentiation via stimulation of sirtuin 1 and AMPK, which is important to maintain vascular plasticity and to adapt against age-dependent vascular changes.[121] Because both sirtuin 1 and AMPK are known regulators of the autophagic process, resveratrol could improve vascular function and prevent the development of cardiovascular diseases via the induction of autophagy. Metformin, on the contrary, is a standard drug for the treatment of type 2 diabetes mellitus, and has been shown to attenuate neointima formation by inhibiting SMC proliferation, migration, and inflammation. Metformin stimulates autophagy via AMPK, which impairs tumor progression,[123,124] but it remains to be determined whether the macrovascular effects of metformin in diabetes mellitus are autophagy dependent.

Although there have been no reports of a deleterious outcome associated with specific autophagy upregulation in vivo,[3] it is important to know that some autophagy-inducing drugs reveal off-target effects that may worsen vascular disease. The toll-like receptor 7 ligand imiquimod is an immunomodulating agent that induces autophagy by improving the interaction between Beclin 1 and the myeloid differentiation primary response gene 88, thereby reducing the binding of Beclin 1 to Bcl-2.[125] However, stimulation of toll-like receptor 7 is also involved in nuclear factor-κB activation and cytokine production. Accordingly, local administration of imiquimod in cholesterol-fed rabbits does not result in stabilization of atherosclerotic plaques, as observed for rapalogs, but triggers inflammation and plaque progression.[126] Moreover, some well-known autophagy inducers (eg, lithium chloride) do not induce autophagy in the vessel wall, but stimulate apoptosis,[127] which is detrimental for the stability of advanced atherosclerotic plaques.

Conclusions

Basal autophagy is an essential in vivo process mediating proper vascular function. It is stimulated by stress-related stimuli in the arterial wall to protect ECs and SMCs against cell death and the initiation of vascular disease, in particular atherosclerosis. Besides its role in cell survival, autophagy regulates SMC phenotype and proliferation. However, during aging autophagy is impaired, as for example in advanced atherosclerotic plaques. Therefore, pharmacotherapy with compounds that stimulate the prosurvival effects of autophagy in the vasculature is an emerging treatment option. Several animal and clinical studies have indicated that mTOR inhibitors effectively inhibit the mechanisms that control atherosclerotic plaque growth and destabilization. However, it is not yet proven whether these effects are mediated via autophagy induction. mTOR inhibitors, such as rapamycin/rapalogs, have a wide margin of safety and may be suitable for daily oral administration. Nevertheless, it is important to note that the adverse effects of these compounds represent major clinical challenges that remain to be addressed in the context of their use for vascular disease. Combination therapy with a statin might guarantee successful atherosclerotic plaque stabilization with minimal adverse effects (Figure 3). The hyperlipidemia associated with mTOR inhibition can be counteracted by the statin, whereas an mTOR inhibitor may reduce the residual risk of cardiovascular disease in statin-treated patients. Because mTOR inhibitors might lead to the onset of diabetes mellitus, combination therapy with metformin is another interesting option. The UK Prospective Diabetes Study showed that metformin has a beneficial effect on cardiovascular disease,[128] possibly through mTORC1 inhibition. Clinical trials are ongoing to evaluate a new generation of ATP-competitive inhibitors that inhibit mTOR kinase activity by competing with ATP for binding to the mTOR kinase domain. This new generation of autophagy inducers might offer better perspectives for safe, long-term treatment of atherosclerosis. Moreover, alternative approaches, such as inhibition of histone deacetylases, are currently explored to stimulate autophagic activity in cardiovascular disease.[129] However, it is important to note that in contrast to the protective capacities of autophagy, excessive autophagy induction might lead to maladaptive effects and even cell death (autosis).[15] Therefore, the dosing should be carefully set to avoid excessive cell loss. Taken together, we are hopeful that future research will provide new drugs or combinations of drugs that fully take advantage of the beneficial effects of autophagy induction in atherosclerosis and other vascular diseases.

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Disclosures

None.

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