BJP British Journal of Pharmacology

DOI:10.1111/bph.13052
www.brjpharmacol.org

REVIEW

Autophagy: an emerging therapeutic target in vascular diseases

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Received
9 July 2014
Revised
27 November 2014
Accepted
7 December 2014

Autophagy is a cellular catabolic process responsible for the destruction of long-lived proteins and organelles via lysosome-dependent pathway. This process is of great importance in maintaining cellular homeostasis, and deregulated autophagy has been implicated in the pathogenesis of a wide range of diseases. A growing body of evidence suggests that autophagy can be activated in vascular disorders such as atherosclerosis. Autophagy occurs under *basal* conditions and mediates homeostatic *functions* in cells but in the setting of pathological states up-regulated autophagy can exert both protective and detrimental functions. Therefore, the precise role of *autophagy* and its relationship with the progression of the disease need to be clarified. This review highlights recent findings regarding autophagy activity in vascular cells and its potential contribution to vascular disorders with a focus on atherogenesis. Finally, whether the manipulation of autophagy represents a new therapeutic approach to treat or prevent vascular diseases is also discussed.

Abbreviations

3-MA, 3-methyladenine; 4-HNE, 4-hydroxynonenal; 7-KC, 7-ketocholesterol; AGE, advanced glycation end product; AMPK, AMP-activated protein kinase; ATG, AuTophaGy-related genes; CVD, cardiovascular disease; EC, endothelial cell; ER, endoplasmic reticulum; MAP1-LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; oxidized LDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; SMCs, smooth muscle cells; VSMC, vascular SMC

Tables of Links

TARGETS	
Catalytic receptors[a]	**Enzymes**[c]
CD40	AMPK
Integrin, αM subunit (CD11b)	ATG4B
RTP Type C (CD45)	Cathepsin D
TLR7	ERK
VEGFR-2	JNK
Transporters[b]	JNK
ABCA1	p38MAPK
	ULK1

LIGANDS	
4-hydroxynonenal (4-HNE)	Metformin
Acrolein	Methylglyoxal (MGO)
Carbamazepine	Osteopontin (OPN)
CD40L	PDGF-BB
Chemerin	Phosphatidylethanolamine
Dexamethasone	Phosphatidylserine
Everolimus	Rac1
Huntingtin	Rapamycin
Imiquimod	Simvastatin
IL-1β	TNF-α
IFN-γ	VCAM-1
Lithium	Valproic acid
Lysophosphatidylcholine	von Willebrand factor

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Autophagy is a 'housekeeping' subcellular process for lysosome-mediated turnover of damaged proteins and organelles first discovered by Christian De Duve in 1963 (De Duve, 1963). Autophagy is ubiquitous in eukaryotic cells, being highly conserved from yeast to human. Three major forms of autophagy have been described: macroautophagy, microautophagy and chaperone-mediated autophagy. Of these, the most prevalent and common form is macroautophagy. This review will focus on macroautophagy, hereafter referred to as autophagy. In this process, the cytoplasmic structures targeted for destruction are sequestered within double-membrane vesicles called autophagosomes and delivered to the lysosome by fusion for breakdown and possible recycling of the resulting macromolecules.

Although autophagy is generally considered to be non-specific, other intracellular components have been suggested to be selectively targeted by autophagy. Under specific conditions, mitochondria, endoplasmic reticulum (ER), peroxisomes, ribosomes, lipid droplets and bacterial pathogens could be sequestered and degraded by autophagosomes (He and Klionsky, 2009; Dong and Czaja, 2011; Youle and Narendra, 2011; Huang and Brumell, 2014). Under physiological conditions, autophagy has an essential homeostatic role by releasing nutrients from macromolecules and by eliminating unwanted constituents from the cell. Autophagy can also be stimulated by stressful conditions including starvation; the degradation of cytoplasmic materials generates amino acids and fatty acids that are used to produce ATP for promoting cell survival (Levine and Yuan, 2005). Besides acting as a cell protector, autophagy participates in embryonic development (Cecconi and Levine, 2008), differentiation (Mizushima and Komatsu, 2011), longevity (Rubinsztein *et al.*, 2011) and immunity (Ma *et al.*, 2013). However, autophagy dysfunction is correlated with diverse pathologies, such as neurodegeneration, cancer, infection and ageing, and also with vascular disorders, including myocardial ischaemia and reperfusion, cardiomyopathy/heart failure, and atherosclerosis (Boya *et al.*, 2013). Despite remarkable progress in this domain, the regulation and functional significance of autophagy in human diseases are still not well defined and, depending on the context, autophagy may act as both a protective and detrimental process.

In this review the current knowledge on the role of autophagy in vascular diseases, with a focus on atherosclerosis, is discussed and the therapeutic potential of manipulating autophagy as a treatment for vascular disorders addressed.

The molecular machinery of autophagy

The details of the autophagic machinery have been already extensively described in several recent reviews (Feng *et al.*, 2014). Therefore, only the major components of the autophagy machinery for understanding the basic concept of autophagy will be described here (Figure 1). The process of autophagy consists of four sequential steps ending with the degradation of cytosolic 'cargo' in lysosomes: initiation and nucleation of phagophore (isolation membrane), expansion of autophagosomes, maturation of autophagosomes into autolysosomes, and the execution of autophagy (final degradation). Autophagy is tightly regulated by more than 30 highly conserved genes called ATG (AuTophaGy-related

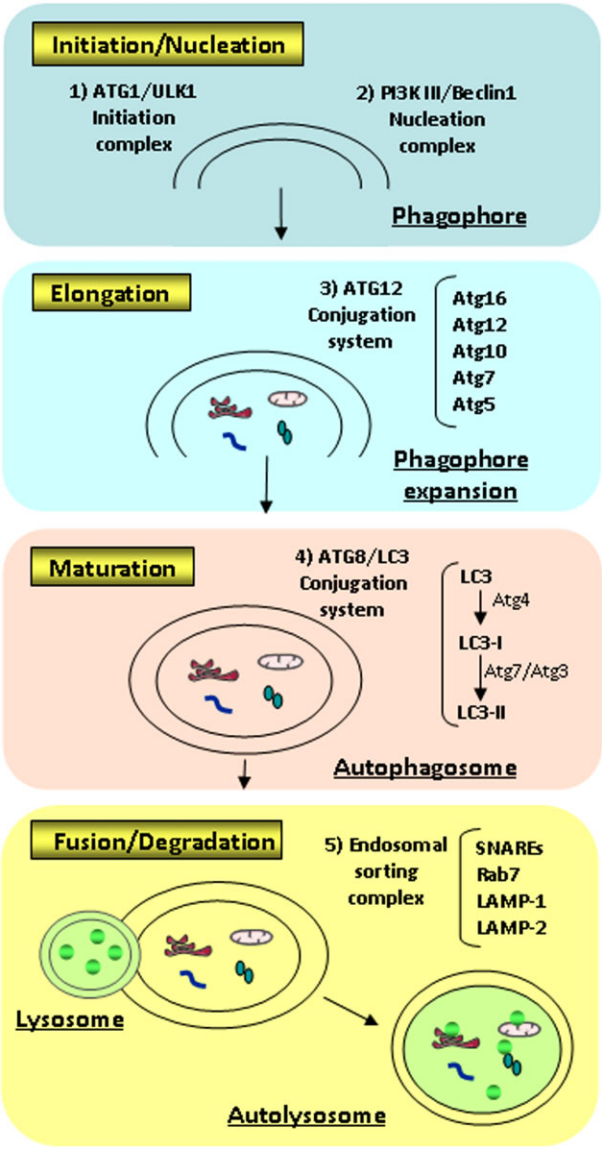

Figure 1

Overview of the autophagy machinery. Once activated, autophagy proceeds through four sequential steps, each step requiring specific regulatory proteins and complexes. Autophagy stimuli lead to the formation of two important complexes, Atg1/ULK1 and PI3K III/Beclin1, which are necessary for the initiation/nucleation step. During this step, phagophore structures are formed from plasma or organellar membranes, the double-lipid bilayer expands and wraps cytoplasmic materials yielding a closed multi-lamellar organelle termed autophagosome. Two ubiquitin-like conjugation systems are part of the elongation and maturation steps. One system involves the covalent conjugation of Atg12 to Atg5 with the help of the E1-like enzyme Atg7 and the E2-like enzyme Atg10. The Atg12–Atg5 conjugate in turn associates non-covalently with Atg16. The presence of Atg16 is required for the localization of Atg5 and Atg12 to the phagophore. The second system involves the conjugation of phosphatidylethanolamine to LC3/Atg8 by the sequential action of Atg4, Atg7 and Atg3. Lipid conjugation leads to the conversion of the soluble form of LC3-I to the autophagosome-associated form LC3-II. The autophagosome undergoes fusion with a late endosome or lysosome, to create an autolysosome, in which sequestered materials are degraded by lysosomal enzymes.

genes) that were initially characterized in *Saccharomyces cerevisiae* (Tsukada and Ohsumi, 1993; Thumm *et al.*, 1994; Harding *et al.*, 1996; Klionsky *et al.*, 2003), followed by the discovery of their mammalian orthologues (Mizushima *et al.*, 2011). Once activated, autophagy begins with the formation of the phagophore (a precursor of autophagosomes), the origin of which is a subject of considerable debate. Several recent data suggest a multi-membrane source model for the biogenesis of autophagosome in mammalian cells: the ER (Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009; Yla-Anttila *et al.*, 2009a), the outer membrane of the mitochondrion (Hailey *et al.*, 2010), the ER-mitochondrial junction (Hamasaki *et al.*, 2013), clathrin-coated vesicles from the plasma membrane (Ravikumar *et al.*, 2010; Moreau *et al.*, 2011), early endosomes (Longatti *et al.*, 2012) and vesicles budding from the ER and Golgi (Hamasaki *et al.*, 2003; Zoppino *et al.*, 2010; Guo *et al.*, 2012). In a very recent study, Ge *et al.* (2013) identified the ER-Golgi intermediate compartment as the most efficient membrane substrate for the biogenesis of the phagophore, thus integrating these two putative sources. Two major essential complexes regulate the recruitment of specific proteins into newly forming autophagosomal membranes. The first one requires the class III PI3K Vps 34 which recruits the autophagy-specific proteins (Atg17, Atg13) in the region of phagophore formation. This macromolecular complex can also contain Beclin1 (the mammalian orthologue of yeast Atg6), p150 Vsp15 (p150), Atg14 or Ambra1. The second complex involved in the early steps of autophagy involves ULK1 (also called Atg1) which interacts with Atg5, Atg12, Atg16, Atg13 and the focal adhesion kinase family-interacting protein of 200 kD (FIP200). The elongation of membranes for the formation of the autophagosome requires two ubiquitin-like conjugating systems. The Atg12-Atg5-Atg16L system : Atg12 is conjugated to Atg5 by Atg7 which is similar to an E1 ubiquitin-activating enzyme and Atg10 is similar to an E2 ubiquitin-conjugating enzyme. Then the conjugated Atg12–Atg5 complex interacts with Atg16L and this complex associates with phagophores localized to the outer membrane of nascent autophagosomes, but it dissociates before the autophagosome is formed. The second ubiquitin-like reactions involve the microtubule-associated protein 1 light chain 3 (MAP1-LC3/Atg8/LC3), the cytosolic form of LC3. LC3-I is generated by the cleavage of pro-LC3 by ATG4B. LC3-I is then conjugated to the lipid phosphatidylethanolamine by Atg7 and Atg3 to form LC3-II (Ravikumar *et al.*, 2010). Since LC3-II is specifically associated with autophagosomes, the level of LC3-II is correlated with the number of autophagosomes and is considered as an indicator of autophagosome formation (Tanida *et al.*, 2008). The mature autophagosomes traffic along microtubules to endosomes or lysosomes using the dynein-dynactin complex, the fusion of autophagosomes with endosomes/lysosomes appears to be mediated by an endosomal sorting complex required for transport, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), GTPase Rab7 proteins and with the lysosomal-associated membrane proteins, LAMP-1 and LAMP-2. In the final step of the autophagy process, the encapsulated 'cargo' is degraded by lysosomal proteases and released (Mizushima, 2007). Therefore, each step between autophagic processes should be tightly regulated for efficient autophagic degradation.

Autophagy in atherosclerosis

Despite recent advances in medical and interventional therapies, cardiovascular diseases (CVDs) continue to be the leading cause of death worldwide. Atherosclerosis is, by far, the main cause of most CVDs. It is a progressive, complex disease often associated with the ageing process and recognized risk factors such as hypercholesterolaemia, hypertension, diabetes and cigarette smoking. Atherosclerosis involves the build-up of fibrous and fatty deposits called plaque inside the arteries. It can affect all of the arteries, but particularly those that supply blood to the heart (coronaries), the neck arteries that supply blood to the brain (carotids), and the arteries that supply the legs (peripheral) (Lusis, 2000). The disease develops through several stages, ultimately ending with a complex plaque accumulated in the artery wall that impedes blood flow. Acute clinical manifestations such as myocardial infarction or stroke are the result of rupture or ulceration of an 'unstable' atherosclerotic plaque.

A large number of studies involving analysis of angiographic data and histological assessment of ruptured plaques have indicated that the composition rather than plaque size or stenosis severity plays a critical role in plaque rupture and thrombosis (Falk *et al.*, 1995). Therefore, today's challenges are the early detection of rupture-prone or so-called vulnerable plaque and the development of strategies that achieve plaque stabilization. Most of the advanced plaques are composed of a 'fibrous cap' consisting of vascular smooth muscle cells (VSMCs) and extracellular matrix that encloses a lipid- and macrophage-rich necrotic core. For example, unstable plaques contain a higher portion of inflammatory cells and lipids, and a lower proportion of VSMC compared with stable lesions (Finn *et al.*, 2010). Vulnerable plaques are also characterized by the accumulation of apoptotic cells and defective phagocytic clearance (efferocytosis), resulting in the lipid-filled necrotic core (Moore and Tabas, 2011).

The mechanisms involved in plaque stability and plaque rupture are rather complex and the oxidizing and inflammatory environment generated by the presence of pro-atherogenic factors [low-density lipoprotein (LDL) and oxidized lipids, oxidative stress, cytokines] can trigger prosurvival and prodeath processes, which are concomitantly activated in cells. The outcome (life vs. death) depends on the balance between these pathways. In addition to apoptosis, there is a growing body of evidence showing that autophagy occurs in developing atherosclerotic plaques (Martinet and De Meyer, 2009). However, in many cell settings, autophagy and apoptosis are often activated by the same stimuli, and share identical effectors and regulators (Codogno and Meijer, 2005; Maiuri *et al.*, 2007). Thus, given the importance of the stage-specific consequences of apoptosis in atherosclerotic lesions and the intricate interplay between apoptosis and autophagy, there is no doubt that autophagy could play a crucial role in plaque progression.

Detection of autophagy in atherosclerotic lesions

Strong evidence for the presence of autophagy features in atherosclerotic lesions is limited and its occurrence is probably not appreciated and underestimated. Although detection guidelines have recently been established for monitoring autophagy in higher eukaryotes (Klionsky *et al.*, 2012), the detection of autophagy in tissue is still difficult to evaluate due to technical limitations. Transmission electron microscopy (TEM) is recognized as the most accurate method to assess autophagy in tissue allowing the visualization of double-membraned autophagic structures; however, this method is time consuming and not appropriate for daily routine (Yla-Anttila *et al.*, 2009b). Martinet *et al.* (2013) recently evaluated the feasibility and specificity of immunohistochemical assessment of macroautophagy-related marker proteins such as LC3, Atg5, CTSD/cathepsin D, Beclin1 or p62/SQSTM1. From their study, they concluded that only LC3 detection is suitable for monitoring autophagy; nevertheless, its staining in the different organs tested (liver, heart, kidney and gut) required a high-quality isoform-specific antibody coupled to a signal amplification system and overexpression of LC3 (e.g. by GFP-LC3 mice). Therefore, when genetic manipulation or other *in vitro* techniques are not feasible, TEM remains the gold standard method for *in situ* evaluation of macroautophagy in human tissue samples (Martinet *et al.*, 2013). Several studies have reported that TEM analysis of dying VSMC of both human and cholesterol-fed rabbit atherosclerotic plaques exhibit certain features of autophagy, such as vacuolization, formation of myelin figures and the inclusion of cytoplasmic ubiquitin (Kockx *et al.*, 1998; Martinet *et al.*, 2004; Jia *et al.*, 2006). A recent report that provided a complete ultrastructural documentation of the autophagic process in human atherosclerotic plaques definitively confirmed that all the major cell types [smooth muscle cells (SMCs), macrophages and endothelial cells (ECs)] found in the lesion may undergo autophagic activation (Perrotta, 2013). However, this analysis did not address whether these observations on human atherosclerotic plaques were at a lesion-specific stage (early vs. more complicated plaques). The marker proteins of autophagy, such as LC3-II, SQSTM1/p62 and Beclin1, have also been detected by immunoblot and immunofluorescence microscopy analysis in human plaques (Martinet *et al.*, 2007) and in mouse models of atherosclerosis (Martinet *et al.*, 2007; Liao *et al.*, 2012; Razani *et al.*, 2012). Although murine models are currently the most extensively used for atherosclerosis studies, caution must be taken when extrapolating mechanisms to human disease because representative lesions in mice models often consist of lipid-laden intimal macrophages without a well-developed fibrous cap or necrosis, both seen in chronic human atherosclerosis. Additionally, intraplaque haemorrhage (IPH) in human plaques, which is a significant factor in necrotic core expansion, is rarely observed in mice (Getz and Reardon, 2012).

Autophagic stimuli in vascular cells

Several *in vitro* studies have demonstrated that autophagy can be induced by various pro-atherogenic stimuli in vascular cells (Table 1).

Table 1
Autophagic stimuli in vascular cells

Stimuli	Setting/cell type	References
TNF-α	Human atherosclerotic plaque/SMC	Jia *et al.*, 2006
Chemerin	Angiogenesis/EC	Shen *et al.*, 2013
Osteopontin	Abdominal aortic aneurysm/SMC	Zheng *et al.*, 2012
PDGF	Phenotype transition/SMC	Salabei *et al.*, 2013
Shh	Neointimal artery lesion/SMC	Li *et al.*, 2012
Oxidized LDL	Apoptotic cell phagocytosis/EC	Muller *et al.*, 2011a
	LOX-1 down-regulation/EC	Nowicki *et al.*, 2007
	Mouse atherosclerotic lesion/SMC	Ding *et al.*, 2013
4-HNE/acrolein/POVPC	Removal of aldehyde-modified protein/SMC	Hill *et al.*, 2008
7-KC	Mouse atherosclerotic lesion/SMC	He *et al.*, 2013
	Human atherosclerotic lesion/SMC	Martinet *et al.*, 2004
AGE	Cell proliferation/SMC	Hu *et al.*, 2012
MGO	Angiogenesis/EC	Liu *et al.*, 2012
Hypoxia	Inhibition of cell proliferation/SMC	Lee *et al.*, 2011b; Ibe *et al.*, 2013

MGO, methylglyoxal; POVPC, 1-palmitoyl-2-oxovaleroyl phosphatidylcholine.

Induction of autophagy by cytokines

Inflammatory cytokines, such as INF-γ and TNF-α, and CD40-CD40-L interactions can induce autophagy in particular settings or conversely suppress it (Levine and Yuan, 2005; Deretic, 2011; Levine *et al.*, 2011; Maiuri *et al.*, 2013). TNF-α, which is secreted by inflammatory cells and SMCs in atheromas, was shown to increase vacuolization and the expression of LC3-II and Beclin1 in SMCs isolated from human atherosclerotic plaques (Jia *et al.*, 2006). Other cytokines such as osteopontin (OPN), a protein involved in vascular inflammation, are able to induce autophagosome formation, the up-regulation of LC3 protein and autophagy-related genes, leading to VSMC cell death in abdominal aortic aneurysms (Zheng *et al.*, 2012). Since inhibition of the integrin/CD44 and p38 MAPK-signalling pathways prevented OPN-induced autophagy, the authors concluded that OPN stimulates autophagy directly through the integrin/CD44 and p38 MAPK-mediated pathways in SMCs. Interestingly, the adipokine chemerin contributes to human aorta EC angiogenesis through the up-regulation of autophagic activity (Shen *et al.*, 2013). Because chemerin is associated with obesity and metabolic syndrome, the potential role of chemerin-induced autophagy in the neovascularization of atherosclerotic lesions needs to be further explored.

Induction of autophagy by reactive lipids

Reactive oxygen species (ROS) (Scherz-Shouval and Elazar, 2011; Lee *et al.*, 2012), oxidized LDL and secondary products of the oxidative degradation of lipids have all been implicated in the activation of autophagy. Treatment of vascular ECs (Nowicki *et al.*, 2007; Muller *et al.*, 2011a) and SMCs (Ding *et al.*, 2013) with oxidized LDL triggers an increase in autophagy-related proteins and in autophagosome formation. Interestingly, exposure of SMCs to modest amounts of highly oxidized LDL (10–40 $\mu g \cdot mL^{-1}$) enhances autophagy and apoptosis, whereas exposure to higher concentrations ($\geq 60\ \mu g \cdot mL^{-1}$) induces high levels of apoptosis and impairs autophagy, indicating that the stress response evoked by autophagy becomes defective when a threshold of cell injury is reached. The oxidative degradation of lipids in lipoproteins leads to the generation of bioactive lipid intermediates and peroxidation end products (Esterbauer *et al.*, 1992). Reactive lipid species such as free aldehydes [e.g. 4-hydroxynonenal (4-HNE), acrolein] and to a lesser extent lipid hydroperoxides (e.g. 1-palmitoyl-2-oxovaleroyl phosphatidylcholine) cause a robust increase in LC3-II, and electron micrographs of 4-HNE-treated SMCs show extensive vacuolization, pinocytic body formation, crescent-shaped phagophores and multilamellar vesicles (Hill *et al.*, 2008). Likewise, human SMCs and mice macrophages exposed to 7-ketocholesterol (7-KC), one of the major oxysterols present in atherosclerotic plaques, display signs of ubiquitination and features of the autophagy process (Martinet *et al.*, 2004; Liao *et al.*, 2012). Recently, He *et al.* (2013) investigated the molecular mechanism by which 7-KC induced autophagy in human SMCs. Their study demonstrated that 7-KC increases Nox4-mediated ROS production, which triggers autophagy in SMC by inhibiting ATG4B activity.

Induction of autophagy by advanced glycation end products (AGEs) and hypoxia

Driven by hyperglycemia and oxidative stress, the formation of AGEs has a pathophysiological role in the development and progression of different oxidative-based diseases including diabetes, atherosclerosis and neurological disorders (Giacco and Brownlee, 2010). Their putative role in the induction of autophagy has been recently demonstrated in vascular cells. AGE-promoted autophagy was shown to con-

tribute to cell proliferation through ERK, JNK and p38 activation in rat aortic SMCs, thus suggesting that the AGE-autophagy pathway can accelerate the development of atherosclerosis in diabetic patients (Hu *et al.*, 2012). Angiogenesis impairments in diabetic peripheral vasculature contribute to the delayed wound healing, the exacerbated peripheral limb ischaemia and even cardiac mortality in diabetic patients. Methylglyoxal, a highly reactive α-oxoaldehyde, reduces endothelial angiogenesis through peroxynitrite ($ONOO^-$)-dependent and autophagy-mediated VEGFR-2 protein degradation, which may represent a mechanism for diabetes-impaired angiogenesis (Liu *et al.*, 2012).

Atherosclerotic plaques develop intraplaque neovascularization, which is a typical feature of hypoxic tissue (Sluimer *et al.*, 2008), and mice deficient in the autophagic protein Beclin1 display a pro-angiogenic phenotype associated with hypoxia (Lee *et al.*, 2011a). Interestingly, in human cultured pulmonary vascular cells exposed to hypoxia, autophagy activation inhibits the hypoxic proliferation of these cells. Moreover, hypoxia has been shown to activate autophagy through the metabolic sensor AMPK in human pulmonary SMCs and the suppression of AMPK expression prevents hypoxia-mediated autophagy and the induction of cell death (Ibe *et al.*, 2013). Nevertheless, how hypoxia contributes to the induction of autophagy in atherosclerotic lesions remains to be determined.

Induction of autophagy by growth factors

Vascular injury and chronic arterial diseases result in exposure of vascular SMCs to increased concentrations of growth factors. As a consequence, SMCs develop a highly proliferative and synthetic phenotype. Treatment of vascular SMCs with PDGF or sonic hedgehog (Shh) increases the expression of the synthetic phenotype markers and promotes autophagy, as assessed by LC3-II abundance, LC3 puncta formation and TEM (Li *et al.*, 2012; Salabei *et al.*, 2013). Autophagy mediated by PDGF or Shh is involved in the proliferation of SMCs and its pharmacological inhibition by 3-MA appears to prevent arterial restenosis. However, the mechanisms involved in growth factor-promoted autophagy need to be further elucidated.

Functional role of autophagy in atherosclerosis: friend or foe?

The functional role of autophagy in vascular diseases is currently under intense investigation and studies have characterized this process both *in vitro* and *in vivo*. Given that increases in autophagy have been observed in various CVDs, a key unanswered question is whether autophagy is protective or harmful in vascular pathology. Both beneficial and detrimental functions have beeen assigned to autophagy during atherosclerosis progression (Martinet and De Meyer, 2009). Recent data have shed light on the protective role of macrophage autophagy in the regulation of atherosclerotic plaque development. Using ApoE-null mice, a well-established model to study atherogenesis, Razani *et al.* (2012) showed that the autophagy markers p62/SQSTM1 and LC3 are mainly colocalized with plaque leukocytes (CD45 positive cells) and monocyte macrophages (CD11b, MOMA-2 positive cells). Interestingly, autophagy became defective in progressing atherosclerotic plaques from ApoE-null mice as assessed by the accumulation of the p62/SQSTM1 in atherosclerotic aortas. Moreover, in ApoE-null mice completely lacking macrophage autophagy, enhanced plaque formation was observed and this led to macrophage inflammasome hyperactivation accompanied by increased IL-1β production. The putative link between defective autophagy and activation of inflammasome could involve different mechanisms: (i) an increase in ROS production due to impaired mitophagy, since release of ROS from damaged mitochondria can activate inflammasome (Naik and Dixit, 2011; Nakahira *et al.*, 2011), or (ii) the accumulation of dysfunctional lysosomes due to phagocytosed cholesterol crystals (Masters *et al.*, 2011). However, recent data have shown that the induction of lysosomal biogenesis blunts the lysosomal dysfunction and inflammasome activation in macrophages isolated from atherosclerotic plaques, even in the absence of autophagy, thus supporting the involvement of additional mechanisms (Emanuel *et al.*, 2014).

Similarly, the group of Tabas has provided additional evidence for the protective role of macrophage autophagy (Liao *et al.*, 2012). They explored how autophagy inhibition affects both apoptosis and phagocytic clearance (efferocytosis) in Atg5-deficient macrophages exposed to oxidative/ER stressors and in advanced atherosclerotic lesions. They showed that defective macrophage autophagy led to increased apoptosis and oxidative stress in advanced lesional macrophages, promoted plaque necrosis and worsened efferocytosis in Atg5-deficient macrophage/LDLR-null mice. The mechanism involved in defective efferocytosis of autophagy-inhibited apoptotic macrophages has not been fully elucidated, but the authors hypothesized that defective autophagy impairs the recognition and internalization of apoptotic cells by phagocytes perhaps by decreasing the expression of cell surface recognition molecules. This makes sense since dying cells lacking the autophagy genes, Atg5 or Beclin1 in embryoid bodies, fail to express the 'eat-me' signal, phosphatidylserine (PS), and secrete lower levels of the 'come-get-me' signal, lysophosphatidylcholine (Qu *et al.*, 2007). In support of these data, we found that vascular ECs silenced for Beclin1 and exposed to oxidized LDL exhibit less PS externalization and uptake by phagocytic macrophages (Muller *et al.*, 2011a). Given the importance of efferocytosis in preventing plaque rupture, further investigations are necessary to establish why autophagy and efferocytosis fail during lesion progression.

Interestingly, the protective function of autophagy against atherosclerosis has been also linked with cholesterol metabolism and lipophagy. Indeed, lipid droplets can be delivered to lysosomes through autophagy, thus facilitating the hydrolysis of cholesterol esters and subsequent ABCA1-mediated cholesterol efflux (Ouimet *et al.*, 2011). These findings were corroborated in Wip1-deficient mice. The deletion of the Wip1 phosphatase, a known negative regulator of Atm-mTOR-dependent signalling, resulted in activated autophagy, suppression of macrophage conversion into foam cells and prevention of atherosclerotic plaque formation (Le Guezennec *et al.*, 2012). The regulation of cholesterol efflux and autophagy via Wip1 may provide the basis to design

novel therapeutic strategies for efficient cholesterol removal from foam cells, and thereby reduce lipid load in early atherosclerotic plaques.

Besides the protective role of macrophage autophagy in atherosclerotic plaque development, autophagy plays an important role in preserving vascular endothelial function by reducing oxidative stress and inflammation and increasing NO bioavailability (LaRocca *et al.*, 2012). The activation of ECs by oxidized LDL with the subsequent increase in endothelial permeability occurs in the early stage of atherosclerosis. Hence, the molecular mechanisms linking autophagy to endothelial dysfunction involve the degradation of oxidized LDL through the autophagic lysosome pathway as demonstrated by the colocalization of DiI-labelled oxidized LDL with LC3 and LAMP-2 (Zhang *et al.*, 2010).

In addition, vascular ECs exposed to oxidized LDL undergo autophagy activation and phagocytic signal exposure through a common mechanism involving Beclin1 (Muller *et al.*, 2011b). Therefore, it is conceivable that autophagy is actually anti-atherogenic, by favouring the processing of oxidized LDL and the clearance of pro-thrombotic apoptotic cells. Interestingly, endothelial secretion of von Willebrand factor required for platelet adhesion to the injured vessel wall is altered in mice with an endothelial specific deletion of Atg7 although these animals have normal vessel architecture and capillary density (Torisu *et al.*, 2013). In the context of IPH, autophagy may have a beneficial role against hemin-induced EC death by clearing the mitochondrial proteins modified by lipid peroxidation (Higdon *et al.*, 2012). Overall, these observations suggest that modulating the autophagic flux may be a useful strategy for preventing thrombotic events.

The general consensus is that successful autophagy of damaged components protects plaque cells against oxidative stress and promotes cell survival. Loss of SMCs contributes to the thinning of the fibrous cap which results in plaque destabilization and rupture (Clarke *et al.*, 2006). Several reports have pointed to the beneficial role of SMC autophagy. Martinet *et al.* (2004; 2008) showed that aortic SMC death induced by low concentrations of statins was reduced by 7-KC-induced autophagy. Similarly, a recent study demonstrated that the up-regulation of autophagy by 7-KC is protective and could be mediated by Nox4-induced ROS production (He *et al.*, 2013). Inhibition of autophagy enhanced both cell apoptosis and necrosis; in contrast, the autophagy inducer rapamycin inhibited cell death of SMCs overloaded with an excess of free cholesterol (Xu *et al.*, 2010). Furthermore, autophagy may be an important mechanism for the survival of vascular SMCs under conditions associated with excessive lipid peroxidation, since autophagy was shown to remove aldehyde-modified proteins, and inhibition of autophagy precipitates cell death in aldehyde-treated SMCs (Hill *et al.*, 2008). Mechanistically, how autophagy suppresses SMC death programmes is not fully understood. One possible mechanism could involve JNK-dependent ER stress activation, since the inhibition of ER stress with the chemical chaperone 4-phenylbutyric acid prevents JNK phosphorylation and autophagy (Haberzettl and Hill, 2013). In contrast, He *et al.* (2013) demonstrated that 7-KC-triggered autophagy prevents SMC death by suppressing the ER stress-apoptosis pathway, and the up-regulation of autophagy by rapamycin exhibited opposite effects. However, these discrepancies could be explained by the nature of the stimuli. 4-HNE, which is known to covalently modify proteins, has been found to promote the carbonylation of ER-sensor proteins such as protein disulfide isomerase, glucose-regulated protein 78; thereby causing unfolded protein response/ER stress and JNK activation (Haberzettl and Hill, 2013). Conversely, the inhibition of ER stress by 7-KC-induced autophagy could result from enhanced intracellular ROS, leading to ATG4B inhibition, thereby promoting autophagy; however, the molecular mechanisms underlying this process require further investigation (He *et al.*, 2013). Another potential mechanism could involve the autophagic removal of damaged mitochondria (also called mitophagy), thus limiting the release of pro-apoptotic proteins such as cytochrome c.

As mentioned above, autophagy is predominantly considered as a protective mechanism in atherosclerosis; however, overwhelming stress and excessively stimulated autophagy may cause the autophagic death of SMCs (Levine and Yuan, 2005) leading to reduced collagen synthesis, thinning of the fibrous cap and ultimately to plaque destabilization. Similarly, the autophagic death of ECs may increase vascular permeability and platelet aggregation, which enhance the risk of thrombosis and acute clinical events (Martinet and De Meyer, 2009). Interestingly, a novel role for autophagy in regulating VSMC phenotype has been recently uncovered. Treatment of vascular SMCs with PDGF-BB which promotes the development of the synthetic vascular SMC phenotype is a robust inducer of autophagy as assessed by LC3-II abundance, LC3 puncta formation and electron microscopy (Salabei *et al.*, 2013). Inhibition of autophagy blocked the degradation of contractile proteins and prevented the hyperproliferation and migration of SMCs, thus supporting the view that autophagy is required for PDGF-induced phenotype conversion and could have a detrimental role in the setting of restenosis. However, future studies are necessary to identify the signalling pathway by which growth factors such as PDGF activate the autophagic programme.

Pharmacological modulation of autophagy in vascular diseases

Pharmacological approaches to modulate autophagy have currently gained increasing attention in the treatment of CVDs. Several drugs that have the potential to inhibit or stimulate autophagy have already been identified (Fleming *et al.*, 2011), and now ongoing clinical trials are testing their association with cytotoxic drugs in a variety of cancers (Cheng *et al.*, 2013). Activators of autophagy (Table 2), for instance, rapamycin and its derivatives (everolimus) that trigger autophagy through the inhibition of mTOR (mammalian target of rapamycin), have been evaluated as potential plaque stabilizing drugs. Local stent-based delivery of everolimus in atherosclerotic plaques from cholesterol-fed rabbits led to a striking reduction in macrophage content without altering SMCs (Verheye *et al.*, 2007). *In vitro* studies showed that treatment of macrophages and SMCs with everolimus induced the inhibition of *de novo* protein synthesis in both

Table 2

Pharmacological modulation of autophagy in the context of atherosclerosis

Compound	Pharmacological	References
Inducer		
Everolimus	Macrolide, rapamycin derivative, mTOR inhibitor	Verheye *et al.*, 2007
Simvastatin	Statin, HMG-CoA reductase inhibitor	Wei *et al.*, 2013
Imiquimod	Imidazoquinoline, Toll-like receptor 7 ligand	De Meyer *et al.*, 2012
z-VAD-fmk	Fluoromethylketone, pan-caspase inhibitor	Martinet *et al.*, 2006
Valproic acid	Carboxylic acid, myo-inositol-1-phosphate synthase inhibitor	Dai *et al.*, 2013
Trehalose	Disaccharide, chemical chaperone	LaRocca *et al.*, 2012
Inhibitor		
Spautin-1	Fluorobenzylquinozaline, deubiquitinases USP10 and USP13 inhibitor	Salabei *et al.*, 2013
3-Methyladenine	Purine derivative, class III PI3K inhibitor	Salabei *et al.*, 2013
Bafilomycin A1	Macrolide antibiotic, vacuolar-type H+-ATPase inhibitor	Salabei *et al.*, 2013

The pharmacological characteristics and mode of action of selected compounds that have been shown to modulate autophagy in the context of atherosclerosis are presented.

cell types by dephosphorylating the downstream mTOR target p70 S6 kinase. The inhibition of translation promoted selective macrophage death and was characterized by bulk degradation of long-lived proteins, processing of LC3 and cytoplasmic vacuolization, which are all markers of autophagy. The authors proposed that the macrophage selectivity is most likely due to the elevated metabolic activity of macrophages that makes them more sensitive to protein synthesis inhibitors than SMCs; however, protein translation inhibition can render SMCs less sensitive to cell death due to contractile-to-quiescent phenotype transition. Hence, because macrophage efferocytosis and autophagy flux decreases as atherosclerosis progresses (Liao *et al.*, 2012; Razani *et al.*, 2012), the clearance of lesional macrophages in the vascular wall via everolimus-induced autophagy could be a promising strategy to promote stable plaque phenotype.

Although mTOR inhibitors have been shown to attenuate plaque progression in atherogenic models, they also enhance macrophage cholesterol efflux and reverse cholesterol transport. A previous report demonstrated that sirolimus treatment for 12 weeks specifically reduces the cholesterol content of the aortic arch of ApoE-null mice compared with untreated mice (Basso *et al.*, 2003). In support of the latter, two recent studies (Ouimet *et al.*, 2011; Le Guezennec *et al.*, 2012) have demonstrated that autophagy plays a role in the hydrolysis of stored cholesterol droplets in macrophages, thus facilitating cholesterol efflux. Nevertheless, therapy with mTOR inhibitors is associated with side effects such as hypercholesterolaemia and hyperglycemia, which are not compatible with plaque stabilization (Martinet *et al.*, 2014). Because statins lower plasma cholesterol and have been shown to induce autophagy via AMPK activation (Zhang *et al.*, 2012) and/or Rac1-mTOR signalling (Wei *et al.*, 2013), the combination of mTOR inhibitors with statin therapy would be beneficial to potentiate mTOR inhibitor-induced autophagy and to prevent unstable plaques. Furthermore, hyperglycaemia could be manageable with the anti-diabetic drug metformin that lowers blood glucose levels but also triggers AMPK activation through mTOR inhibition (Liao *et al.*, 2011). Therefore, the development of a new generation of mTOR inhibitors with limited off-target effects would undeniably enhance their efficiency to treat vascular diseases.

Autophagy can also be modulated through mTOR-independent pathways, albeit with different outcomes on plaque phenotype as described previously. Macrophages express Toll-like receptors (TLRs) that recognize pathogens and eliminate intracellular pathogens by inducing autophagy. Local administration of a TLR7 ligand imiquimod in atherosclerotic plaques of cholesterol-fed rabbits induced macrophage autophagy without affecting SMCs (De Meyer *et al.*, 2012). Surprisingly, autophagy activation via imiquimod was detrimental because it was associated with cytokine release, up-regulation of VCAM-1, infiltration of T-cells and plaque progression. The deleterious effect of imiquimod could be explained by its ability to activate NF-κB which could repress autophagy. Although treatment with dexamethasone suppressed these pro-inflammatory effects *in vivo*, caution must be taken since TLR7 stimulation could play a role in promoting atherosclerosis by activating dentritic cells homing to atherosclerotic vessels (Doring *et al.*, 2012; Macritchie *et al.*, 2012). Several other drugs can induce autophagy by an mTOR-independent pathway, mainly by the regulation of inositol-1,4,5-triphosphate (IP_3) levels, but whether these drugs affect macrophage cell fate or other cell types in the plaque is currently unknown. Carbamazepine, valproic acid and lithium increase the intracellular clearance of misfolded protein accumulation through induction of autophagy by reducing the intracellular levels of IP_3 (Williams *et al.*, 2002; Sarkar *et al.*, 2005). Interestingly, stimulation of autophagy by valproic acid decreases calcification by reducing matrix vesicle release in vascular SMCs (Dai *et al.*, 2013). Additionally, using a cell-based screening method, several calcium channel blockers (CCBs) and antiarrhythmic drugs, such as verapamil, loperamide, amiodarone, nimodipine, nitrendipine, niguldipine and pimozide, have been identified as autophagy inducers by inhibiting

intracellular levels of calcium (Fleming *et al.*, 2011). Previous studies have shown that CCBs have anti-atherogenic effects beyond their BP-lowering effects. Their pleiotropic actions in vascular cells involve, for instance, suppression of ROS and inflammation, inhibition of SMC proliferation and migration or activation of peroxisome proliferator-activated receptor gamma (PPAR-γ), but whether these effects are linked to the induction of autophagy has not presently been determined and certainly needs to be investigated further. The pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk) has the ability to induce autophagy and necrotic cell death in macrophages and, indirectly, necrosis of vascular SMCs based mainly on the differential expression of receptor-interacting protein 1 (Martinet *et al.*, 2006). Consequently, a caspase inhibitor may have a detrimental effect due to stimulation of inflammatory responses and, indirectly, SMC necrosis.

Trehalose, a disaccharide present in many non-mammalian species (Sarkar *et al.*, 2007), enhances the clearance of autophagy substrates such as mutant huntingtin and A53T α-synuclein, which are associated with Huntington's disease and familial Parkinson's disease. Trehalose supplementation restores the expression of autophagy markers and rescues vascular endothelial function by increasing NO bioavailability, reducing oxidative stress and normalizing inflammatory cytokines in arteries of ageing mice (LaRocca *et al.*, 2012). Advancing age is a major risk factor for CVD, therefore autophagy-enhancing strategies may have therapeutic efficacy for ameliorating age-associated arterial dysfunction.

A few studies have described a beneficial role of pharmacological inhibition of autophagy in vascular diseases. Recently, Salabei *et al.* (2013) revealed that autophagy plays a role in the contractile-to-synthetic VSMC phenotype transition induced by growth factors. Autophagy inhibition by three pharmacological unrelated inhibitors, such as 3-methyladenine, spautin-1 or bafilomycin A1, stabilized the contractile phenotype. The remarkable efficiency of spautin-1 *in vitro* suggests that it might be a useful therapeutic agent for preventing the phenotype switching and proliferation that occur in vascular injury, such as restenosis.

Conclusion and future challenges

In conclusion, there is mounting evidence showing that autophagy plays a critical role in vascular diseases such as atherosclerosis. Although many autophagic-specific genes and the basic molecular machinery of autophagy have now been well characterized, a first challenge is to identify more selective pharmacological compounds that target unique molecular effectors/regulators of autophagy to specifically modulate the process. Similarly, it is also crucial to establish which of the four sequential autophagy steps should be preferentially targeted to develop a successful autophagy-based therapy.

Currently, the pharmacological modulation of autophagy by blocking mTOR function has shown beneficial effects on plaque phenotype. An alternative approach to circumvent their side effects will be to explore compounds that control autophagy downstream of the mTOR complex, for instance, the Beclin1 complex or the ubiquitin-like conjugation systems. However, caution must be taken when enhancing autophagosome formation if impaired lysosome activity also takes place with the disease. The consequences of the accumulation of autophagosomes in the cytosol could be detrimental for the cell.

A second challenge is to achieve a definite understanding of the autophagy process at all stages of the atherosclerotic lesion. Indeed, the relevance of beneficial autophagy in the early stages and a dysfunctional autophagy observed in the late stages of mouse atherosclerotic models remains to be demonstrated in human clinical samples before we can consider targeting autophagy in the treatment of vascular diseases. Moreover, the favourable effects of mTOR inhibitors on preventing the early stages of atherogenesis, such as monocyte recruitment, macrophage accumulation and SMC phenotypic modulation require further investigation to prove their effectiveness on the restoration of autophagy in advanced lesions.

Given the central role of macrophages in atherosclerotic plaque destabilization, the selective clearance of lesional macrophages in atherosclerotic plaques via drug-induced autophagy is a hopeful strategy. However, chronic or excessive periods of autophagy can have detrimental consequences for the cell and ultimately lead to inflammation and cell death. Therefore, a third challenge is how to accurately activate beneficial autophagy in a selective manner without inducing aberrant cell death or inflammation. For instance, new attractive therapies based on cell specific-targeted nanoparticles and bioabsorbable drug-eluting scaffolds could be used to deliver relevant autophagy modulator compounds to atherosclerotic lesions with reduced side effects.

Overall, in view of the fundamental importance of autophagy in many cellular functions, the pharmacological modulation of autophagy undoubtedly represents a promising tool for the treatment of vascular diseases.

Acknowledgements

C. V. is supported by INSERM and grants from La Fondation de France, La Fondation Coeur et Recherche, Association Française contre les Myopathies/Téléthon and La Fédération Française de Cardiologie. Dr Frank Lezoualc'h is thanked for his critical reading of the manuscript.

Conflict of interest

None.

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