Autophagy in the cardiovascular system

Guido R.Y. De Meyer *, Wim Martinet

Division of Pharmacology, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

1. Introduction

Macroautophagy (further referred to as autophagy) is a dynamic and highly regulated process of self-digestion. The hallmark of autophagy is the de novo formation of autophagosomes, which are double-membrane vacuoles originating from a largely undefined structure, known as the phagophore or isolation membrane. The process shows elongation of the isolation membrane, maturation of the autophagosome followed by its fusion with a lysosome, thereby generating an autophagolysosome or autolysosome. The incorporation of the outer autophagosomal membrane with the lysosomal membrane eventually allows the degradation of the remaining inner single-membrane and the cytoplasmic content of autophagosome by lysosomal hydrolases. Autophagy is a highly conserved cellular process responsible for the removal or recycling of long-lived proteins and organelles, which also provides cells with an alternate source of nutrients from the reutilization of cellular proteins and organelles. This lysosomal degradation pathway is essential for survival, differentiation, development, and homeostasis. In eukaryotic cells, autophagy occurs constitutively at low levels to perform housekeeping functions such as the destruction of dysfunctional organelles.

Upregulation occurs in the presence of external stressors (e.g. starvation, hormonal imbalance, oxidative stress) and internal needs (e.g. removal of protein aggregates), suggesting that the process is an important survival mechanism. Indeed, autophagy principally serves an adaptive role to protect organisms against diverse pathologies [1], including heart disease [2]. However, in certain circumstances, the self-cannibalistic function of autophagy may be deleterious [3]. Several lines of evidence indicate that autophagy is associated with heart disease, cancer and a number of neurodegenerative disorders such as Alzheimer’s, Parkinson’s and Huntington’s disease. In addition, autophagy plays a role in development, aging and immunity [4–6].

2. Autophagy in the normal heart and in heart disease

One of the first reports that described autophagy in the heart was published in the mid 1970s, one decade after the initial description of autophagy in mammalian cells [7]. Sybers et al. [8] observed that fetal mouse heart in culture continues to beat for a period of weeks, but that degenerative changes occur. Electron microscopy revealed formation of autophagic vacuoles containing damaged organelles in some cells after the first day, indicating focal cytoplasmic injury. This process was accelerated by transient deprivation of oxygen or glucose. At present, several lines of evidence indicate that autophagy is indeed essential for cellular homeostasis in the heart, maintaining cardiac structure and function [9,10]. Autophagy under baseline conditions...
has a housekeeping role in the turnover of cytoplasmic constituents. A
defect in this pathway will result in adverse effects for the heart. For
instance, severe cardiac dysfunction occurs in patients and mice
showing defective autophagic degradation owing to a deficiency of
the lysosomal-associated membrane protein-2 (LAMP-2) [11,12]. This
disorder, also known as Danon's disease [13,14], is a lysosomal
glycogen storage disease characterized by cardiomyopathy, myopathy
and variable mental retardation. Mutations in the coding sequence of
LAMP-2 cause a LAMP-2 deficiency and extensive autophagy in
skeletal and heart muscle.

While autophagy is an ongoing process in baseline conditions, it is
more apparent in disease. Increased autophagic activity in cardio-
mocytes has been described after multiple forms of cardiovascular
stress, including starvation, chronic ischemia, infarction–reperfusion
injury, pressure overload, cardiomyopathy, and heart failure [15–20].
Whether autophagy functions as a pro-survival or pro-death program
during disease is still not completely understood [21]. Autophagy can
have both beneficial and detrimental roles in the myocardium,
depending on the level of autophagy. Upregulation of autophagy
may be beneficial to the cell by recycling of proteins to generate free
amino acids and fatty acids needed to maintain energy production,
by removing damaged organelles, and by preventing accumulation of
protein aggregates. However, enhanced autophagy can also contribute
to cell death, possibly through excessive self-digestion. Numerous
autophagosomes are often seen in dying cells, but it is not clear
whether autophagy directly contributes to cell death or is upregulated
as an effort to prevent it.

2.1. Autophagy removes protein aggregates in the heart

Cardiomyocytes face enormous challenges to correctly fold nascent
desmin-related cardiomyopathy and is an adaptive response to
accelerates heart failure progression with an increase in interstitial
fibrosis, greater accumulation of polyubiquitinated proteins, larger
and more extensive intracellular aggregates, accelerated ventricular
dysfunction, and early mortality. Thus, autophagy is also activated in
desmin-related cardiomyopathy and is an adaptive response to
protein aggregates in this proteotoxic form of heart disease [27].

2.2. Autophagy removes damaged organelles in the heart

Besides removal of toxic protein aggregates, autophagy can also
provide protection to the heart by removing damaged and dysfunc-
tional organelles. In particular, the removal of leaky mitochondria
releasing pro-apoptotic factors such as cytochrome c [28] and
apoptosis inducing factor [29] may protect cells by preventing
activation of apoptosis. Many studies have described mitochondria
sequestered inside autophagosomes in the myocardium after cellular
distress [8,29,30]. Sybers et al. [8] noted numerous autophagosomes
containing mitochondria in fetal hearts in organ culture after hypoxia/
reoxygenation. Decker and Wildenthal [30] also observed that many
autophagic vacuoles contained damaged mitochondria during reper-
fusion and proposed that autophagy is upregulated to remove
damaged mitochondria. Since the mitochondrial permeability transi-
tion pore opens when reperfusion is initiated after ischemia [31], it
might serve as a signal to autophagosomes to sequester the
mitochondria. Furthermore, the mitochondrial protein Bnip3 (Bcl-2/
adenovirus E1B 19 kDa interacting protein) is upregulated in
cardiomyocytes subjected to ischemia and stimulates apoptotic cell
death signaling during ischemia/reperfusion (I/R) injury of the heart
via disruption of mitochondrial integrity, which in turn leads to
enhanced superoxide production and the release of pro-apoptotic
factors. Bnip3 activation is associated with upregulation of autophagy
as determined by high levels of autophagosomes containing frag-
mented mitochondria. Upregulation of autophagy most likely con-
stitutes a protective response against Bnip3 death signaling by
removing harmful and leaky mitochondria, thus preventing activation
of apoptosis (Fig. 1) [29].

Ablerrant protein aggregation in the form of pre-amyloid oligomers
(PAOs) has been observed in failing mouse and human hearts [24].
Indeed, cardiomyocyte expression of a polyglutamine (PQ) PAO causes
heart failure [25,26]. Intracellular PAOs cause toxicity in many of the
protein misfolding-based neurodegenerative diseases. For example, in
Huntington's disease, long (>50) PQ repeats form PAOs and cause
neurotoxicity, whereas shorter PQ peptides are benign. In transgenic
mice, hearts expressing an 83 residue-long PQ repeat (PQ83) show
reduced cardiac function and dilation by 5 months, and mice die by 8
months. In contrast, control mice, expressing non-amyloid-forming
peptide of 19 PQ repeats, have normal cardiac function, morphology,
and life span. PQ83 protein accumulates within aggresomes with PAO-
specific staining. The PQ83 hearts exhibit increased autophagosomal
and lysosomal content but also show markers of necrotic death, including
inflammatory cell infiltration and increased sarcolemmal permeability.
Thus, protein misfolding resulting in intracellular PAO accumulation
is sufficient to cause cardiomyocyte death and heart failure.

Furthermore, a severe form of desmin-related cardiomyopathy is
also characterized by accumulation of misfolded proteins [24]. This
disease is triggered by a missense mutation in the alphaB-crystallin
(CryAB) gene. Mutant CryAB (CryAB[R120G]) induces an increase in
cardiomyocyte autophagic activity [27]. Blunting autophagy increases
the rate of aggregate accumulation and the abundance of insoluble
CryAB[R120G]-associated aggregates. Cardiomyocyte–restricted over-
expression of CryAB(R120G) in mice induces intracellular aggregate
accumulation and systolic heart failure by twelve months. Well before
the earliest decline in cardiac function, significant autophagic activity
is present. Blunting autophagy in vivo, through crossbreeding CryAB
(R120G) mice with animals harboring heterozygous inactivation of
beclin 1, a protein required for early autophagosome formation,
accelerates heart failure progression with an increase in interstitial
fibrosis, greater accumulation of polyubiquitinated proteins, larger
and more extensive intracellular aggregates, accelerated ventricular
dysfunction, and early mortality. Thus, autophagy is also activated in
desmin-related cardiomyopathy and is an adaptive response to
protein aggregates in this proteotoxic form of heart disease [27].
induction of autophagy through inhibition of mTOR [32,37] (Fig. 1), 
AMPK is activated [32,36]. AMPK activation probably triggers 
the initiation and maturation of autophagy [35]. mTOR is a serine/
rapamycin (mTOR) dependent and independent pathways regulate 
after I/R, whereas extended ischemia correlates with irreversible 
correlates with functional recovery and salvage of the myocardium 
and promote survival of cardiac cells. Thus, the increase in autophagy 
generates free amino acids and fatty acids, which can be 
protective. Indeed, degradation of proteins and organelles by 
the ubiquitin–proteasome system, which is activated during oxidative 
stress. The combination of upregulation of beclin 1 and downregulation of Bcl-2 during 
the reperfusion phase stimulates the activity of beclin 1, thereby stimulating autophagic 
cell death. Thus, it seems that autophagy in patients should be stimulated in the middle 
of ischemia but must be inhibited during reperfusion.

2.3. Autophagy protects the heart during ischemia but may become detrimental during reperfusion

Autophagy may promote survival by maintaining energy homeostasis during ischemia. Although induction of autophagy during the ischemic phase is protective, further enhancement of autophagy during the reperfusion phase may induce cell death and appears to be detrimental [32], as explained below.

2.3.1. Autophagy during ischemia

Langendorff perfused rabbit hearts subjected to I/R show signs of autophagy induction [30,33]. Twenty minutes of ischemia does not induce autophagy, but the number of autophagosomes increases 
when reperfusion is initiated. However, 40 min of ischemia alone 
causes an increase in autophagy which is further enhanced during reperfusion. When ischemia is extended to 60 min, large and likely 
dysfunctional lysosomes are present during reperfusion, suggesting 
that prolonged ischemia impairs the autophagic-lysosomal pathway. 
Under ischemia, oxygen and nutrient supplies are decreased and the 
intracellular generation of ATP via oxidative phosphorylation is reduced. These stimuli induce autophagy [34], which may be protective. Indeed, degradation of proteins and organelles by autophagy generates free amino acids and fatty acids, which can be 
used to maintain mitochondrial ATP production and protein synthesis 
and promote survival of cardiac cells. Thus, the increase in autophagy 
correlates with functional recovery and salvage of the myocardium 
after I/R, whereas extended ischemia correlates with irreversible 
damage and contractile dysfunction [30]. Both mammalian targets of 
rapamycin (mTOR) dependent and independent pathways regulate 
the initiation and maturation of autophagy [35]. mTOR is a serine/threonine kinase and a member of the PI kinase-related family that 
controls the response to changes in nutrients. Ischemia induces 
cardiac autophagy on condition that AMP-activated protein kinase (AMPK) is activated [32,36]. AMPK activation probably triggers 
induction of autophagy through inhibition of mTOR [32,37] (Fig. 1), 
followed by phosphorylation of eukaryotic elongation factor-2 and 
inhibition of protein synthesis [37,38].
2.4. Autophagy modulates cardiac hypertrophy and the transition from hypertrophy to heart failure

In the setting of hemodynamic stress, such as occurs in hypertension or following myocardial infarction, the heart undergoes a compensatory hypertrophic growth response. Left unchecked, this hypertrophic response triggers myocyte death, ventricular dilation, diminished contractile performance, and a clinical syndrome of heart failure. For some years, autophagy has been implicated in the pathophysiology of heart failure [50]. For example, in isolated neonatal myocytes, inhibition of autophagy by knockdown of Atg7 using RNAi induces hypertrophy, and conditional deletion of Atg5 in the heart results in increased cross-sectional area of the myocytes [14]. On the other hand, the mTOR inhibitor rapamycin, a potent activator of autophagy, prevents cardiac hypertrophy induced by thyroid hormone treatment [51] or aortic banding [52]. Rapamycin treatment can even regress already established cardiac hypertrophy induced by pressure overload and improves cardiac function [53]. Thus, autophagy might antagonize cardiac hypertrophy by increasing protein degradation which would decrease cardiac mass.

In contrast, increased autophagy in hypertrophied hearts may also play a role in the transition from stable cardiac hypertrophy to decompensated heart failure [54]. Pressure overload induced by aortic banding evokes heart failure and greatly increases cardiac autophagy. Load-induced autophagic activity peaks at 48 h and aortic banding evokes heart failure and greatly increases cardiac autophagy.

Deterimental

- Protection against oxidative stress → anti-apoptosis
- Protection against inflammation, hypoxia, metabolic stress
- SMC autophagy in fibrous cap low levels → plaque instability
- Persistent oxidative stress → apoptosis
- Ceroid impairs autophagy → apoptosis
- SMC autophagy in fibrous cap high levels → plaque instability
- EC autophagy high levels → thrombosis

Autophagy in atherosclerosis

- Remains significantly elevated for at least 3 weeks. In addition, increased autophagy in hypertrophied hearts may also play a role in the transition from stable cardiac hypertrophy to decompensated heart failure [54]. Pressure overload induced by aortic banding evokes heart failure and greatly increases cardiac autophagy. Load-induced autophagic activity peaks at 48 h and remains significantly elevated for at least 3 weeks. In addition, autophagic activity is not spatially homogeneous but rather is seen at particularly high levels in basal septum. Heterozygous disruption of the gene coding for beclin 1 decreases cardiomyocyte autophagy and diminishes pathological remodeling induced by severe pressure stress. Conversely, beclin 1 overexpression increases autophagic activity and accentuates pathological remodeling (hypertrophy upon pressure overload). Taken together, these findings implicate autophagy in the pathogenesis of load-induced heart failure and suggest that it may be a target for novel therapeutic intervention [54]. Furthermore, the level of autophagy in the heart is an important factor in determining whether autophagy will be protective or detrimental. By giving intramuscular injections of diphtheria toxin, Akazawa et al. [55] observed degeneration of cardiomyocytes within 7 days in transgenic mice that express human diphtheria toxin receptor in the heart. Approximately 80% of the animals showed pathophysiological features characteristic of heart failure and were dead within 14 days. Degenerated cardiomyocytes of the transgenic heart showed several characteristics indicative of autophagic cell death such as upregulation of lysosomal markers and accumulation of autophagosomes. Moreover, the high levels of autophagy observed in the failing heart support the theory that excessive induction of autophagy underlies autophagic cell death and loss of cardiomyocytes (Fig. 2). Indeed, dead and dying cardiomyocytes showing characteristics of autophagy have been reported in heart failure caused by dilated cardiomyopathy [15,16,56], valvular and hypertensive heart disease [57], chronic ischemia [17], and stunned or hibernating myocardium [18,58], but not in normal heart [16,18]. The incidence of autophagic cardiomyocytes in failing hearts is greater than the incidence of apoptotic cells (0.03–0.3% versus ≤0.002%, based on stainings for granular ubiquitin inclusions or TUNEL, respectively [15,16,18]). Therefore, autophagy is suggested to be an important mechanism underlying the cardiomyocyte dropout responsible for the worsening of heart failure [59]. However, it remains unclear whether autophagy is a sign of failed cardiomyocyte repair or a suicide pathway for the failing cardiomyocytes [60].

Autophagy in cardiomyocytes is not confined to heart failure, but also occurs in patients suffering from a cardiomyopathy without overt heart failure. For example, Saijo et al. [19] reported a case of cardiomyopathy in which most of the myocytes were affected by
autophagic vacuolization despite a normal cardiac index and the lack of diastolic dysfunction. Autophagic cell death was accompanied by a markedly elevated (600 μg/ml) plasma level of brain natriuretic peptide that might have caused induction of autophagy in the heart. Apart from cardiomyocytes, autophagy also occurs in interstitial cells of the aortic valve of patients with severe aortic valve stenosis [61].

2.5. Autophagy protects against famine and excessive β-adrenergic stimulation of the heart

Autophagy serves as a catabolic energy source in times of famine. Cardiac myocytes from starved mice display high numbers of autophagosomes to survive the adverse conditions of nutrient deprivation [62]. Autophagy in cardiac myocytes has also been suggested to provide a necessary source of energy between birth and suckling [63].

β-adrenergic stimulation, which promotes apoptosis [64] and induces cardiac hypertrophy and heart failure [65], has been reported to inhibit autophagy [66]. Cardiac myocytes isolated from Atg5 deficient mouse heart have increased sensitivity to the β-adrenoceptor antagonist isoproterenol compared to wild type cells [14]. Moreover, isoproterenol treatment for 7 days leads to left ventricular dilatation and cardiac dysfunction in autophagy deficient mice but not in wild type mice, suggesting that autophagy protects cells against excessive β-adrenergic stimulation.

2.6. Lipofuscin is formed via autophagy in the heart and impairs autophagy during aging

Lipofuscin is a nondegradable, yellow-brown pigment composed of lipid and protein residues that progressively accumulates in cardiac myocytes and other long-lived postmitotic cells. Autophagy in the heart is responsible for the formation of lipofuscin [67]. Hydrogen peroxide (H₂O₂) generated by mitochondria and other organelles permeates in the lumen of secondary lysosomes [67]. These lysosomes contain iron derived from cellular structures undergoing autophagic degradation. The interaction between reactive ferrous iron and H₂O₂ results, via fenton reactions, in the generation of hydroxyl radicals inducing lipid peroxidation and eventually intermembrane cross-linking and lipofuscin formation [67]. Although autophagy is a nonstop renewal process responsible for the degradation of damaged organelles and macromolecules, degenerative changes gradually advance in the aging heart, even under favorable conditions [68]. This finding suggests that autophagy is unable to completely remove all damaged structures. Progressive inhibition of autophagy in the aging heart is at least in part attributed to intralysosomal accumulation of lipofuscin. Cross-linked polymeric lipofuscin cannot be degraded by lysosomal hydrolases and might lead to preferential allocation of lysosomal enzymes to lipofuscin-loaded lysosomes at the expense of active autolysosomes [68]. Impaired autophagy stimulates further accumulation of damaged mitochondria, increased reactive oxygen species (ROS) generation and enhanced lipofuscinogenesis [68]. Interestingly, continuous autophagic intralysosomal degradation of ferruginous materials combined with the formation of H₂O₂ and the peroxidation of the lysosomal membrane might result in its subsequent rupture, especially under conditions of oxidative stress, with release of harmful lysosomal enzymes [69]. If of limited magnitude, such release can induce 'reparative autophagy' [70], causing additional accumulation of iron and undegradable oxidation products such as lipofuscin. Finally, these events sensitize cells to undergo apoptosis as released lysosomal enzymes can attack other proteins and mitochondria, triggering cytochrome c release with an amplification of the apoptotic program [69].

Taken together, autophagy can have dual roles in the heart (Fig. 2). Autophagy functions predominantly as a pro-survival pathway during nutrient deprivation and other forms of cellular stress. However, when autophagy is severely triggered, the autophagic machinery may also be used for self-destruction. In this way, autophagic cell death can occur in cardiac cells and finally leads to heart failure. Although it is not known what factors determine whether autophagy will be protective or detrimental to a cell, it is likely that the level and duration of autophagy are important [9]. For instance, low levels of autophagy during ischemia and early reperfusion may protect against cell death by providing the cell with free fatty acids and amino acids and by removing damaged organelles, whereas high levels or long-term upregulation of autophagy during reperfusion can trigger cell death by excess degradation of essential proteins and organelles. In addition, there is a complex interrelationship between autophagy and apoptotic cell death pathways, in which regulators of apoptosis also function as regulators of autophagic activation [9,71]. One can also hypothesize that the activation of protective autophagy might be accompanied by apoptosis. In this situation, depending on the extent of activation of each of these pathways, some cells would show traditional autophagic morphology but would survive, some would show evidence of autophagy but die from apoptosis, while others would die from apoptosis alone. In situations where apoptosis is inhibited, e.g. when ATP depletion is extreme, necrosis might occur [72].

2.7. Therapeutic modulation of autophagy in the heart

Modulation of the autophagic pathway may represent a potential future therapeutic target to treat or prevent a variety of cardiovascular diseases. However, despite the discovery of many autophagy-specific genes and the dissection of signaling pathways involved in the regulation of autophagy, therapeutic approaches to modulate autophagy in cardiovascular disease are highly limited. Several possibilities can explain this discrepancy. First, the few autophagy inhibitors that are currently used in cell culture experiments, in particular the class III phosphoinositide kinase (PI3 K) inhibitor 3-methyladenine, are unsuitable for in vivo applications because of their high toxicity [73]. Second, the most effective inducer of autophagy in mammalian cells is nutrient starvation, a strategy which is obviously not attractive in vivo and even dangerous from a cardiovascular point of view. Intermittent fasting in rats protects the heart from ischemic injury and attenuates post-myocardial infarction cardiac remodeling [74], likely via anti-apoptotic/inflammatory mechanisms and possibly via induction of autophagy, but prolonged starvation triggers severe cardiovascular complications and cardiac death [75]. Several alternative strategies have recently been developed to regulate autophagy in cardiomyocytes. For example, treatment of UM-X71 hamsters, a model of cardiomyopathy and muscular dystrophy that is caused by lack of the δ-sarcoglycan gene [76], with granulocyte colony-stimulating factor significantly improves survival, cardiac function and remodeling in these animals, and such beneficial effects are accompanied by a reduction in autophagy, an increase in cardiomyocyte size, and a reduction in myocardial fibrosis [77]. Moreover, autophagy in cardiac myocytes after I/R is also reduced by the endogenous cardiac peptide urocortin [43] that inhibits Β1 expression. Other compounds that are able to regulate autophagy in the heart include the β-blocker propranolol, the calcium channel blocker verapamil (both have a stimulatory effect) and the β-adrenoceptor agonist isoproterenol that increases CAMP levels (and inhibits autophagy) [66,78]. These effects are in accordance with a recently identified mTOR-independent pathway involving cAMP–Ca²⁺–calpains–Gsx, which leads to induction of autophagy after pharmacological inhibition [79]. Because verapamil, in contrast to propranolol, affects neither the β-adrenergceptors nor the intracellular levels of the second messenger cAMP, it has been suggested that stimulation of autophagy is a regulatory step in the adaptation of the heart to a reduction in cardiac output [78]. The
Fas/Fas-L interactions and the production of cytotoxic compounds. Large lipid core, high macrophage content and a thin myocardial infarction or sudden death. Such plaques have a relatively plaque has major clinical implications. When a plaque develops an leads to the development of plaques in the vessel wall. Recent evidence indicates that not only the size but also the stability of the plaque stability. Indeed, SMCs can produce collagen fibers which contribute to the tensile strength of the fibrous cap. Moreover, autophagy can protect plaque cells against oxidative stress by degrading the damaged material, in particular polarized mitochondria in the very early stages before cytochrome c release occurs [80]. In this way, successful autophagy of the damaged components is anti-apoptotic and contributes to cellular recovery (Fig. 2). However, acute or persistent oxidative stress results in an intracellular increase of ROS that damage the lysosomal membrane [90]. Alteration of the lysosomal compartment prevents fusion with autophagic vacuoles containing damaged components, and results in the release of potent hydroxides, enhancing the degree of cellular damage. If autophagy is not engaged as part of the oxidative stress response in atherosclerotic plaques, or when oxidative injury overcomes the cellular defenses, cells probably die via apoptosis [90] (Fig. 2). The protective role of autophagy in atherosclerosis is also illustrated by the finding that SMC death induced by low concentrations of statins is not stimulated, but attenuated by the autophagy inducer 7-ketocholesterol [91]. Possibly, the engulfment of defective mitochondria by autophagosomes limits the release of pro-apoptotic proteins, such as cytochrome c and apoptosis inducing factor, into the cytosol [92].

Beside its important anti-apoptotic role in atherosclerotic plaques, autophagy may also downregulate apolipoprotein-B (apoB)-containing lipoproteins in the circulation. Retention of apoB-containing lipoproteins within the arterial wall is an important initiating event in the pathogenesis of atherosclerosis. Dietary polyunsaturated fatty acids induce the appearance of intracellular aggregates of apoB in the liver [93,94]. These aggregates slowly degrade by an autophagic process, thereby inhibiting the export of apoB-lipoproteins by hepatocytes and the subsequent infiltration of these compounds in the vessel wall.

3.2. Cereoid that is formed via autophagy in atherosclerotic arteries impairs autophagy and induces apoptosis

Apart from its protective role, autophagy in atherosclerosis is responsible for the formation of cereoid, which is an insoluble complex of protein associated with oxidized lipids found in all human atherosclerotic lesions [95]. The process of cereoid formation is similar to lipofuscinogenesis (see above) and involves severe oxidative stress combined with autophagy. Iron and cereid deposits
colocalize either extracellularly or intracellularly in foam cell-like macrophages or SMCs of advanced plaques [96]. Ceroid deposits cannot be degraded by lysosomal hydrolases and might lead to preferential allocation of the lysosomal enzymes to ceroid-loaded lysosomes at the expense of active autolysosomes which in turn would lead to progressive impairment of autophagy and the induction of apoptosis [69] (Fig. 2).

3.3. Excessively stimulated autophagy may cause autophagic death in plaque cells

In contrast to basal autophagy, excessively stimulated autophagy may cause autophagic SMC death [89], which in turn results in plaque destabilization owing to the reduced synthesis of collagen and thinning of the fibrous cap. Also autophagic death of endothelial cells may be detrimental for the structure of the plaque as endothelial injury and/or death represents a primary mechanism for acute clinical events by promoting lesional thrombosis (Fig. 2). In vitro observations suggest that various atherosclerosis-related factors stimulate autophagy in plaque cells as discussed below.

3.4. Autophagy is stimulated by oxidized lipids and removes harmful oxidatively modified proteins in atherosclerotic plaques

During atherosclerotic plaque formation, low-density lipoproteins (LDL) infiltrate atherosclerosis-prone arterial regions where they are oxidatively or enzymatically modified. Oxidative degradation of oxidized lipids generates several bioactive intermediates and end-products [97], including lipid hydroperoxides and lipid peroxidation-derived aldehydes such as malondialdehyde or 4-hydroxynonenal (4-HNE). Exposure of SMCs to 4-HNE leads to the modification of several proteins and evokes autophagosome formation [98]. Protein modifications by 4-HNE may be harmful not only because they disrupt protein function but also because they lead to the accumulation of inactive or cross-linked proteins, which must be removed to prevent further toxicity. Proteasome-mediated degradation is of minor importance for the removal of these modified proteins [99]. However, protein-aldehyde adducts may be degraded as part of the autophagic response. Indeed, inhibition of autophagy increases the accumulation of protein–4-HNE adducts, whereas the removal of protein–4-HNE adducts is enhanced by the autophagy stimulator rapamycin [98]. 4-HNE treated cells display several characteristics of autophagy, yet the mechanisms by which 4-HNE or protein–4-HNE adducts trigger autophagy are not completely understood. Because oxidative stress is a well-known stimulus of autophagy to facilitate the removal of damaged organelles [90], autophagy may be induced by increased generation of ROS and oxidative injury (Fig. 2). Products of lipid peroxidation could also directly trigger autophagic signaling through formation of electrophile-modified or cross-linked proteins. Autophagy can also be evoked by oxidized LDL (oxLDL) [100]. One of the major oxysterols present in oxLDL, 7-ketocholesterol, not only triggers autophagy are not completely understood. Because oxidative stress mechanisms by which 4-HNE or protein-4-HNE adducts trigger autophagy is enhanced by the autophagy stimulator rapamycin [98]. 4-HNE treated cells display several characteristics of autophagy, yet the mechanisms by which 4-HNE or protein-4-HNE adducts trigger autophagy are not completely understood. Because oxidative stress is a well-known stimulus of autophagy to facilitate the removal of damaged organelles [90], autophagy may be induced by increased generation of ROS and oxidative injury (Fig. 2). Products of lipid peroxidation could also directly trigger autophagic signaling through formation of electrophile-modified or cross-linked proteins. Autophagy can also be evoked by oxidized LDL (oxLDL) [100]. One of the major oxysterols present in oxLDL, 7-ketocholesterol, not only triggers autophagy and removes harmful oxidatively modified proteins in atherosclerotic plaques

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3.5. Autophagy is stimulated by inflammation and removes damaged components in atherosclerotic plaques

Together with monocytes, T-lymphocytes infiltrate the arterial intima at an early stage in atherogenesis and release pro-inflammatory cytokines such as IFN-γ, interleukin (IL)-2 and TNFα [101]. It has been shown that these cytokines stimulate autophagy [102–104]. Given the predominantly pro-inflammatory Th1 type immune response in atherosclerosis [101], inflammatory cells in advanced plaques serve as an important source of pro-autophagic stimuli. Th2 cytokines IL-4 and IL-13 have the potential to act as suppressors of autophagy because they stimulate type I PI3 K and thus also mTOR [102].

In macrophage-derived foam cells, inducible nitric oxide synthase is upregulated leading to the production of cytotoxic amounts of NO [105]. NO may contribute to oxidative stress and tissue damage through formation of peroxynitrite, which can potentially oxidize and/or damage polyunsaturated fatty acids, sulhydryl groups and cellular DNA. This process may evoke an autophagic response to remove the damaged material [90].

3.6. Hypoxia and metabolic stress in atherosclerotic plaques stimulate autophagy to protect cells against severe cellular damage

In advanced human atherosclerotic plaques, inadequate vascularization causes hypoxia and hypoxia-induced cell death [106]. Nutrient starvation induces autophagy [107], whereas metabolic stress (nutrient deprivation combined with hypoxia) leads to damage to organelles, proteins, and DNA that potently stimulates apoptosis [108]. As autophagy is not only an alternate means to generate ATP during starvation, but also maintains homeostasis through protein and organelle quality control, autophagy can mitigate metabolic stress to protect cells against severe cellular damage [109] (Fig. 2). Furthermore, SMCs in the fibrous cap of advanced human plaques are surrounded by a thick layer of basal lamina [110]. Therefore, it is conceivable that autophagy in these caged cells is stimulated as a result of starvation.

3.7. Therapeutic modulation of autophagy in atherosclerosis

Because macrophages play a central role in atherosclerotic plaque destabilization [111], selective induction of macrophage death now gains increasing attention in cardiovascular medicine to stabilize vulnerable, rupture-prone lesions [112]. Compared with apoptosis or necrosis, autophagy seems to be an interesting type of death to eliminate macrophages in atherosclerotic plaques, at least from a theoretical point of view, because autophagic cells literally digest themselves to death. As a consequence, the cytoplasmic content progressively decreases so that activation of inflammatory responses, the release of matrix degrading proteases and the deposition of necrotic debris after postautophagic necrosis is minimal. However, sometimes strategies to clear macrophages in plaques can be hampered by lack of specificity or unexpected adverse effects. For example, the pancaspase inhibitor z-VAD-fmk induces autophagy and necrotic cell death in J774A.1 and RAW264.7 macrophages as well as in IFN-γ primed primary mouse peritoneal macrophages, but not in vascular SMCs or C2C12 myoblasts [113]. Presumably, autophagy acts as a cell survival mechanism to protect against z-VAD-fmk-induced necrotic cell death [114]. z-VAD-fmk-treated J774A.1 macrophages overexpress and secrete several chemo- kines and cytokines, including TNFα [113]. The combination of z-VAD-fmk and TNFα, but not TNFα alone, induces SMC necrosis [113]. In this regard, z-VAD-fmk is detrimental and not beneficial for atherosclerotic plaque stability due to the stimulation of an inflammatory response and indirect induction of SMC death.

3.7.1. Selective depletion of macrophages in atherosclerotic plaques via autophagy induction through inhibition of mTOR-dependent pathways

Inhibition of mTOR by rapamycin or its derivatives (rapalogs) mimics amino acid and growth factor deprivation, and exerts a cytostatic effect on proliferating cells [115]. By blocking proliferation of activated T cells, rapamycin is used as an immunosuppressant in organ transplantation. Its ability to inhibit SMC proliferation and migration is the pharmacological base of its use as a therapeutic agent to prevent restenosis after balloon angioplasty and stenting [115]. In addition to its effects on cell growth, inhibition of mTOR may lead to autophagic cell death through activation and/or upregulation of certain Atg proteins [116]. For example, Atg13 is rapidly dephosphorylated upon inhibition of the mTOR pathway, stimulating its affinity for Atg1. The Atg1–Atg13 association is required for autophagosome formation [117]. Stent-based delivery of the rapamycin derivative everolimus in...
rabbit atherosclerotic arteries leads to autophagic cell death of plaque macrophages through autophagic cell death induction without altering the SMC content [118]. Everolimus inhibits de novo protein synthesis in both macrophages and SMCs by dephosphorylating the downstream mTOR target p70 S6 kinase, followed by bulk degradation of long-lived proteins, processing of microtubule-associated protein light-chain 3 (LC3) and cytoplasmic vacuolization in macrophages but not in SMCs [118]. Interestingly, apart from the mTOR pathway, local administration of the protein synthesis inhibitor cycloheximide also induces selective macrophage death in rabbit atherosclerotic plaques, but in contrast to everolimus, apoptosis and not autophagy is induced [119]. The mechanism for the selective induction of autophagic cell death in macrophages versus SMCs is not completely understood. However, measurements of oxygen consumption [120] as well as immunodetection of markers for DNA synthesis/repair [121] indicate that plaque macrophages are metabolically highly active and consequently more sensitive to protein synthesis inhibitors as compared to SMCs. Moreover, inhibition of translation in SMCs by rapamycin induces a modulation towards a differentiated, quiescent, contractile phenotype, which may render SMCs relatively insensitive to cell death mediated by inhibition of protein translation [122]. Therefore, inhibition of translation rather than differential expression of cell death proteins seems to be the major trigger that drives selective induction of macrophage death.

3.7.2. Selective depletion of macrophages in atherosclerotic plaques via autophagy induction through modulation of mTOR-independent pathways

Autophagy can also be induced in atherosclerotic plaques through modulation of mTOR-independent pathways. For example, lithium, which is an inhibitor of inositol monophosphatase, depletes free inositol and reduces the levels of 1,4,5-inositol triphosphate [123]. Recent experiments in our laboratory showed that treatment with lithium triggers selective macrophage death (I. De Meyer, unpublished data, 2008). Furthermore, the imidazoquinoline compound imiquimod stimulates autophagy after binding to Toll-like receptor 7 (TLR7) [124]. This protein is expressed only in immune cells so that imiquimod is able to induce autophagic death in macrophages, but not in SMCs (I. De Meyer, unpublished data, 2008). However, a clear link between the downstream signaling pathways induced by TLR7 and autophagy cannot be drawn at present. Recently, another mTOR-independent autophagy pathway involving cAMP–Ca²⁺–calpain–Ggα has been described [79]. Pharmacological inhibition of this pathway induces autophagy. This can be achieved by e.g. the L-type Ca²⁺ channel blocker verapamil. Whether this pathway affects macrophages or other cell types in the plaque is presently unknown. Furthermore, small molecule enhancers of rapamycin (SMERs) have recently been identified [125]. Three SMERs induce autophagy independently, or downstream of mTOR. In combination with rapamycin at concentrations saturating for its pro-autophagic activity, these SMERs result in greater rates of autophagy substrate clearance, compared to either the SMERs or rapamycin alone.

Dietary or pharmacological lipid lowering is a well-known approach to clear macrophages in atherosclerotic plaques [126,127]. However, loss of macrophages in rabbit atherosclerotic plaques after lipid lowering seems not to be related to induction of macrophage apoptosis, but is mainly a consequence of impaired monocyte recruitment followed by decreased macrophage replication. Alternatively, monocyte-derived cells can emigrate from the plaque [128] during lesion regression, and depletion of cholesterol may induce autophagy [129], possibly due to disruption of cholesterol-rich lipid rafts. The resultant downregulation of Akt activity [130,131] leads to suppression of mTOR and autophagy induction. However, only drastic cholesterol depletion methods induce autophagy of cells in vitro [129]. Because LDL-levels of cholesterol-fed rabbits do not dramatically change during the first weeks of dietary lipid lowering, we feel that, at least in the first weeks, induction of autophagy is not involved in macrophage clearance after cholesterol withdrawal.

Both cholesterol and plant sterols are present in our diet, but intestinal epithelial cells selectively and efficiently rid the body of plant sterols. However, a rare mutation in plant sterol excretion results in the accumulation of plant sterols in plasma and tissues, leading to sitosterolemia. The excess atherosclerotic heart disease in patients with sitosterolemia may be explained by the observation that excess cholesterol kills macrophages by caspase-dependent apoptosis, whereas sitosterol-induced macrophage death occurs by autophagy and necroptosis [132].

3.8. Future directions for the study of autophagy in atherosclerosis

Crossbreeding of mouse models for atherosclerosis (e.g. ApoE or LDL receptor knockout animals) with autophagy deficient mice (e.g. conditional Atg5 knockout animals) might give further insight whether autophagy is harmful or protective in atherosclerotic plaques. Most likely, autophagy under basal conditions plays an important role in cellular housekeeping, while induced autophagy may function as a death pathway.

Stimulation of survival via autophagy in SMCs of vulnerable atherosclerotic plaques can help to prevent coronary artery syndromes and sudden death. Furthermore, selective clearance of macrophages in atherosclerotic plaques via drug-induced autophagy is a promising approach, but probably only a first step toward plaque stabilization. A combined approach, e.g. together with dietary lipid lowering or statin treatment, may be needed to prevent re-infiltration of macrophages. In this way long-term plaque stabilizing effects might be obtained.

Although pharmacological induction of autophagic cell death in macrophages is believed to be the preferred type of death to deplete this type of cells from atherosclerotic plaques [133], it should be noted that also this approach might involve certain adverse effects. In vitro, autophagic macrophages produce pro-inflammatory cytokines such as TNFα and IL-6 [134], suggesting that the autophagic process is not immunologically silent. Moreover, it remains unclear what happens with the large amount of oxidized lipids in the cytoplasm of macrophage-derived foam cells undergoing autophagy. It is tempting to speculate that lipids in the cytosol are not adequately digested during autophagy owing to overload or exhaustion of lysosomal enzymes. Furthermore, an increase rather than a decrease in foam cell formation can be expected in macrophages undergoing autophagy because protein degradation as well as the decline of protein synthesis in autophagic cells readily blocks the utilization of lipids for lipid–protein conjugation which in turn results in the formation of lipid droplets [133]. These lipid droplets can be spilled out in the microenvironment of the plaque when the autophagic cell collapses, thereby attracting new mononuclear cells from the circulation. Further research is needed before autophagic death of macrophages can be exploited to therapeutic advantage in unstable atherosclerotic plaques.

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