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Review

# Autophagy in the cardiovascular system

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ABSTRACT

Autophagy is a catabolic pathway for bulk turnover of long-lived proteins and organelles via lysosomal degradation. Growing evidence reveals that autophagy is involved in the progression or prevention of many human diseases. Here we discuss the role of autophagy in the normal heart, in heart disease and atherosclerosis. In the heart, autophagy functions predominantly as a pro-survival pathway during cellular stress by removing protein aggregates and damaged organelles, protecting the heart against famine, excessive β-adrenergic stimulation and ischemia. However, when severely triggered, e.g. during reperfusion, the autophagic machinery may lead to cell death. Furthermore, autophagy modulates cardiac hypertrophy and the transition from hypertrophy to heart failure. During aging, lipofuscin is formed via autophagy in the heart and impairs autophagy. Basal autophagy in atherosclerotic plaques is a survival mechanism safeguarding plaque cells against cellular distress, in particular oxidative injury, metabolic stress and inflammation, by removing harmful oxidatively modified proteins and damaged components. Hence, autophagy is anti-apoptotic and contributes to cellular recovery in an adverse environment. However, excessively stimulated autophagy causes autophagic death in plaque cells and is detrimental. Ceroid that is formed via autophagy in atherosclerotic arteries impairs autophagy and induces apoptosis. Basal autophagy can be intensified by appropriate drugs and pharmacological approaches have been developed to stabilize rupture-prone plaques through selective induction of macrophage autophagic death, without affecting the plaque stabilizing smooth muscle cells.



## 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 the 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

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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 cardiomyocytes 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 polypeptides and to keep mature proteins from denaturing. They have developed multi-layered protein quality control mechanisms which are carried out primarily by chaperones and ubiquitin–proteasome system (UPS)-mediated proteolysis. Autophagy also participates in protein quality control in cardiomyocytes, especially under pathological conditions. The UPS is responsible for the degradation of short-lived proteins, such as cytosolic, nuclear, and myofibrillar proteins, whereas autophagy regulates levels of long-lived proteins and organelles [9]. The UPS and autophagy are generally considered to be two separate degradation pathways, but recent findings have demonstrated that there may be cross-talk between the two pathways. Cardiac protein quality control often becomes inadequate in heart disease, which may play an important role in the development of congestive heart failure [22]. Cardiomyocytes with deficient autophagy have increased levels of ubiquitinated proteins in autophagosomes, indicating that autophagy may work in parallel with the UPS to turnover cellular proteins [9]. Moreover, cardiac-specific deficiency of Atg5 leads to cardiac hypertrophy, left ventricular dilatation and contractile dysfunction in adult mice [14]. Atg5-deficient hearts show increased levels of ubiquitination, disorganized sarcomeres and mitochondrial aggregation [14]. However, embryonic Atg5 knockout mice are viable and live to adulthood without any detectable heart abnormalities [14], presumably due to compensatory mechanisms which also perform cellular maintenance. Given that the production of polyubiquitinated proteins is often increased during cardiomyopathy and chronic heart failure [22], it is likely that autophagy provides protection by degrading misfolded proteins and aberrant protein aggregates that may be toxic to cardiomyocytes. Indeed, pressure overload promotes accumulation of polyubiquitinated protein aggregates in the left ventricle, development of aggresome-like structures, and a corresponding induction of autophagy [23]. This finding situates pressure-overload heart disease in the category of proteinopathy. Moreover, attenuation of autophagic activity enhances aggresome size and abundance, which is consistent with a role for autophagic activity in protein aggregate clearance in the heart.

Aberrant 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 overexpression 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].

### 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 dysfunctional 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 reperfusion and proposed that autophagy is upregulated to remove damaged mitochondria. Since the mitochondrial permeability transition 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 fragmented mitochondria. Upregulation of autophagy most likely constitutes a protective response against Bnip3 death signaling by removing harmful and leaky mitochondria, thus preventing activation of apoptosis (Fig. 1) [29].

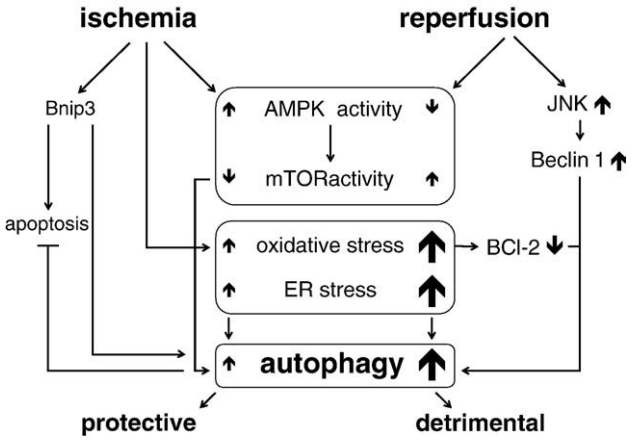


**Fig. 1.** Induction of autophagy in the heart after ischemia/reperfusion. During ischemia, oxygen and nutrient supplies are decreased, causing activation of AMP-activated protein kinase (AMPK) and inactivation of mammalian target of rapamycin (mTOR), which in turn leads to autophagy for cell survival. Furthermore, Bcl-2/adenovirus E1B 19 kDa interacting protein (Bnip3) is involved in upregulation of autophagy and functions as a cytoprotective pathway to oppose ischemia/reperfusion-related apoptosis. Upon reperfusion, activation of AMPK and inactivation of mTOR are no longer observed. Instead, expression of beclin 1 is markedly upregulated. The ability of beclin 1 to promote autophagy is negatively regulated by interaction with Bcl-2. The latter protein is degraded 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].

#### *2.3.2. Autophagy during reperfusion*

During the reperfusion phase, activation of AMPK is no longer observed, but expression of beclin 1 is markedly upregulated [32,36] (Fig. 1). AMPK is rapidly inactivated upon reperfusion, but overexpression of beclin 1 in cardiac myocytes following I/R enhances formation and downstream lysosomal degradation of autophagosomes (autophagic flux) and significantly reduces activation of the pro-apoptotic protein Bax [39]. Moreover, autophagosome formation during reperfusion is significantly inhibited in beclin $1^{+/-}$ mice [36]. The mechanism that drives upregulation of beclin 1 in the heart is presently unknown. Recent evidence suggests that nitric oxide (NO) is not involved in increased beclin 1 expression, though NO plays a crucial role in the process of I/R and heart failure by regulating several members of the caspase family [40]. In vascular smooth muscle cells (SMCs), tumor necrosis factor-α (TNFα) induces upregulation of beclin 1 expression and subsequent autophagy through the JNK pathway [41]. Since JNK is activated by reperfusion [42], beclin 1 may be upregulated by JNK during the reperfusion phase. Simultaneously, increased oxidative stress and ER stress may occur, which may further stimulate autophagy [32] (Fig. 1).

In contrast to the generally protective effects of autophagy during ischemia, autophagy during the reperfusion phase may not necessarily be protective. For instance, inhibition of hypoxia and reoxygenation-induced autophagy leads to enhanced cardiac myocyte survival in vitro [43]. Moreover, inhibition of autophagy during the reperfusion phase is accompanied by significant reduction in the size of myocardial infarction and cardiac myocyte apoptosis in beclin $1^{+/-}$ mice [36]. Several factors could render autophagy detrimental in the heart during reperfusion. First, excessive activation of autophagy could cause cell death. Second, the combination of upregulation of beclin 1 and downregulation of Bcl-2 during the reperfusion phase would stimulate the activity of beclin 1, thereby stimulating cell death. The ability of beclin 1 to promote autophagy is negatively regulated by interaction with Bcl-2 [44] (Fig. 1). The latter protein is degraded by the UPS, which is activated during oxidative stress [45]. Third, autophagy and apoptosis are interconnected by common mediators. E.g. Atg5 interacts with Fas-associated death domain (FADD) protein, an adaptor molecule involved in mediating receptor-mediated apoptosis, and mediates interferon-γ (IFN-γ) induced cell death[46]. Moreover, upregulation of calpain during I/R may convert initial activation of autophagy by Atg5 to apoptosis [47]. Indeed, cleavage of Atg5 by calpain promotes the translocation of truncated Atg5 to the mitochondria where it interacts with Bcl-$X_L$ and triggers permeabilization of the outer mitochondrial membrane [47]. Overexpression of truncated Atg5 induces apoptosis but is unable to promote autophagy. Fourth, selective degradation of catalase may play a role as well [32].

Thus it seems that autophagy in patients should be stimulated in the middle of ischemia but must be inhibited during reperfusion. However, whether induction of autophagy during I/R is beneficial or detrimental may also depend on the extent of the initial ischemia. If the level of ischemia is modest, activation of autophagy at reperfusion may also be modest and thus protective. Interestingly, myocardial protection elicited by adaptation to ischemia is mediated in association with BAG-1 protein (Bcl-2-associated athanogene) [48]. The latter is a multifunctional pro-survival molecule that binds with Hsp70/Hsc70 and enhances the anti-apoptotic effects of Bcl-2. Moreover, hibernation, a reversible arrest of contractile function, is an important energy-saving adaptation to chronic hypoxia that enables cardiomyocytes to withstand severe ischemic insults. Autophagy seems to be a key pro-survival mechanism in hibernating cardiomyocytes [49], which represent the critical reserve of dysfunctional cells that can be potentially rescued. Certainly, more investigations are needed to translate our knowledge regarding autophagy to clinical treatment in order to reduce I/R injury.

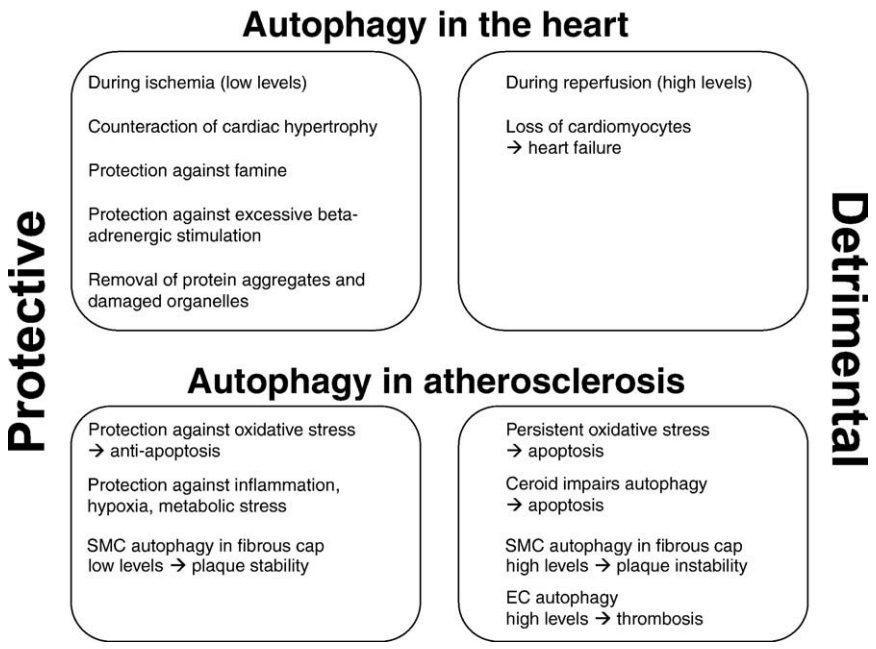


**Fig. 2.** Dual role of autophagy in the heart and in atherosclerosis. During nutrient deprivation and cellular stress, autophagy functions predominantly as a pro-survival pathway in the heart. However, when severely triggered the autophagic machinery may be used for self-destruction, finally leading to heart failure. During ischemia low levels of autophagy protect against cell death by providing the cell with free fatty acids and amino acids and by removing damaged organelles, whereas during reperfusion high levels or long-term upregulation of autophagy trigger cell death. In atherosclerosis, mild oxidative stress activates autophagy to facilitate the removal of damaged organelles. Successful autophagy of the damaged components contributes to cellular recovery. If autophagy is not sufficient for the removal of the cellular damage, e.g. in the case of severe oxidative stress, leakage of intramitochondrial components such as cytochrome *c* may induce apoptosis through activation of the caspase cascade. Moreover, oxidative damage of the lysosomal membrane often results in cytosolic leakage of potent hydrolases, which could cause substantial cytosolic damage followed by apoptosis. Severe oxidative stress combined with autophagy may also lead to formation of ceroid, a complex of protein associated with oxidized lipids. Ceroid deposits cannot be degraded by lysosomal hydrolases and might lead to preferential allocation of lysosomal enzymes to ceroid-loaded lysosomes at the expense of active autolysosomes which in turn would lead to progressive inhibition of autophagy and the induction of apoptosis. Autophagic cell death of smooth muscle cells (SMC) of the fibrous cap may lead to plaque destabilization and autophagic cell death of endothelial cells (EC) may result in thrombosis.

### *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 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 lysomal 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 agonist isoproterenol compared to wild type cells [14]. Moreover, isoproterenol treatment for 7 days leads to left ventricular dilation 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 ($H_2O_2$) 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 $H_2O_2$ results, via fenton reactions, in the generation of hydroxyl radicals inducing lipid peroxidation and eventually intermolecular 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 $H_2O_2$ 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 phosphoinositol 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-X7.1 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 beclin 1 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-adrenoreceptor 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–$Ca^{2+}$–calpains–Gsα, which leads to induction of autophagy after pharmacological inhibition [79]. Because verapamil, in contrast to propranolol, affects neither the β-adrenoreceptors 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

mTOR inhibitor and autophagy inducer rapamycin can also be useful to treat the heart, as it confers preconditioning-like protection against I/R injury in isolated mouse heart through opening of mitochondrial $K_{ATP}$ channels [80]. In addition, rapamycin in low doses (25–100 nM) reduces necrosis as well as apoptosis following simulated ischemia-reoxygenation in adult cardiomyocytes [80], although the direct impact of autophagy in these processes is unclear. As explained above, it is likely that autophagy should be stimulated in the middle of ischemia but must be inhibited during reperfusion in patients (Fig. 1).

Clinical heart failure results from the cumulative loss of functioning myocardium from any cause. Current therapeutics, such as early myocardial revascularization after myocardial infarction, are focused exclusively upon minimizing cardiac myocyte necrosis and may even contribute to secondary apoptosis and autophagy. Two pro-apoptotic proteins, in particular Bnip3 and Nix, are transcriptionally upregulated specifically in response to myocardial ischemia and pathological hypertrophy and have been examined as therapeutic targets. In Bnip3 and Nix genetic mouse models, prevention of cardiac myocyte apoptosis in ischemic and hemodynamically overloaded hearts salvaged myocardium, minimized late ventricular remodeling, and enhanced ventricular performance. Cardiomyocyte resuscitation, i.e. bringing cardiac myocytes that are destined to die back to life by preventing programmed cell death, shows promise as an additional approach to minimizing cell death for long-term prevention of heart failure [81].

### 2.8. Future directions for the study of autophagy in heart disease

The most fundamental question for autophagy in heart disease is whether its role is harmful or protective. Accumulation of protein aggregates in cardiomyocytes occurs in response to either hemodynamic stress or genetic mutation of critical proteins, but experimental evidence shows that autophagy is maladaptive in one context and beneficial in the other. It is not known whether there are inherent differences in the types of protein aggregates that form and/or the associated autophagic response. Some evidence suggests that increased autophagic activity does not directly clear aggregates themselves but rather clears aggregate precursors, shifting the equilibrium away from aggregate formation [82]. The pathophysiological outcome may be determined by severity and/or duration of the autophagic response or the nature of the autophagic substrate. This dose- and context-dependent role of autophagy in heart disease poses special challenges [83]. At present, it is not known how long one can activate autophagy without detrimental consequences for the cell. Moreover, there is no good explanation for the discrepancy between the fact that autophagic cell death in cardiomyocytes of patients suffering from heart failure is only observed in a minority of cells, while the functional impact is often dramatic. In addition, we do not know whether senescent SMCs or long-living cells such as cardiomyocytes are less capable of inducing autophagy as compared to other cell types. From a therapeutic point of view, the challenge for clinicians will be to selectively turn on autophagy-mediated survival in the treatment of heart disease without activating death pathways.

## 3. Autophagy in atherosclerosis

Atherosclerosis is a chronic inflammatory disease of arteries and 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 has major clinical implications. When a plaque develops an unstable phenotype, it may easily rupture followed by thrombosis, myocardial infarction or sudden death. Such plaques have a relatively large lipid core, high macrophage content and a thin fibrous cap. It is well known that macrophages in the plaque initiate SMC death via Fas/Fas-L interactions and the production of cytotoxic compounds. Moreover, secretion of metalloproteinases by macrophages results in degradation of collagen and thinning of the fibrous cap. As a consequence, it is generally assumed that macrophages play a key role in plaque destabilization and rupture, while SMCs contribute to plaque stability [84]. The role of autophagy in atherosclerosis is still poorly understood. Due to technical and practical limitations for detection [85] but also due to the lack of adequate marker proteins, the occurrence of this process in atherosclerotic plaques has not yet been analyzed in detail [86].

### 3.1. Basal autophagy is a survival mechanism in atherosclerotic plaques and is anti-apoptotic

Disintegrating SMCs in the fibrous cap of experimental or human plaques show certain features of autophagy using transmission electron microscopy, such as severe vacuolization and formation of myelin figures, which are phospholipids and membrane fragments, often arranged in concentric rings, representing autophagic degradation of membranous cellular components. These structures are not abundantly present in human atherosclerosis, but they can be detected in plaques from cholesterol-fed rabbits [87] as well as after treatment of cultured SMCs with oxidized lipids [88]. Since autophagy is often considered as a survival mechanism and not as a death pathway [89], it is likely that autophagy of SMCs in the fibrous cap of advanced plaques is in the first place an important mechanism for 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 [90]. 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 hydrolases, 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. Ceroid 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 ceroid, which is an insoluble complex of protein associated with oxidized lipids found in all human atherosclerotic lesions [95]. The process of ceroid formation is similar to lipofuscinogenesis (see above) and involves severe oxidative stress combined with autophagy. Iron and ceroid 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 infiltrated 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 protein-4-HNE modification and oxidative damage, but also induces signs of autophagy in SMCs [88].

### 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, sulfhydryl 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 chemokines 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–$Ca^{2+}$–calpains–Gsα has been described [79]. Pharmacological inhibition of this pathway induces autophagy. This can be achieved by e.g. the L-type $Ca^{2+}$ 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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