AUTOPHAGY IN HEALTH AND DISEASE: IV. The role of pancreatic beta-cell autophagy in health and diabetes

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Abstract

Autophagy is an evolutionarily conserved machinery for degradation and recycling of various cytoplasmic components such as long-lived proteins and organelles. In pancreatic beta-cells, as in most other cells, autophagy is also important for the low basal turnover of ubiquitinated proteins and damaged organelles under normal conditions. Insulin resistance results in up-regulation of autophagic activity in beta-cells. Induced autophagy in beta-cells plays a pivotal role in the adaptive expansion of beta-cell mass. Nevertheless, it is not clear whether autophagy is protective or detrimental in response to cellular stresses in beta-cells. In this review, we describe the crucial roles of autophagy in normal function of beta-cells and discuss how dysfunction of the autophagic machinery could lead to the development of diabetes mellitus.

Key Words: autophagy; insulin secretion; beta cell; diabetes; FFA
Introduction

Insulin resistance caused by various factors such as obesity and reduced physical activity has long been considered the hallmark of type 2 diabetes mellitus (Kahn 2001). The natural history of diabetes, however, depends largely on the adaptation of pancreatic-β cells to meet the increased demand for insulin secretion resulting from insulin resistance. β-cell failure is currently considered the primary determinant of progression of insulin resistance to diabetes (Prentki and Nolan 2006; Sachdeva and Stoffers 2009). Pancreatic β-cells are specialized cells that secrete insulin. The cytosol of normal β-cells is largely occupied by numerous insulin granules. Beta-cells maintain abundant synthesis of insulin proteins, secreting and degrading them ceaselessly in response to fluctuations in blood glucose level. It has been postulated that β-cells are exposed to various forms of cellular stresses from misfolded proteins or damaged mitochondria, particularly in states of insulin resistance (Hartley et al. 2009; Kaniuk et al. 2007). Identification of the cellular systems that protect β-cells during states of insulin resistance has been one of the goals for establishing strategies to achieve long-standing glycemic control in diabetes. Recent in vivo studies, including ones from our group indicate that macroautophagy (here referred to as autophagy), a protein degradation system, plays an important role in the maintenance of normal β-cell function and survival (Ebato et al. 2008; Jung et al. 2008). On the other hand, increased levels of autophagosomes can correlate with β-cell death under certain circumstances. In this review, we discuss the role of autophagy in pancreatic β-cells and its possible implication in the pathogenesis of diabetes.
Autophagy as a bulk degradation system

To adjust to fluctuations in external nutritional supplies, eukaryotic cells carry unique systems for recycling their own constituents through lysosome-mediated degradation. Exposure of the cells to a nutritional stress, starvation, triggers the formation of a cap shaped-double-membrane structure, called the isolation membrane, in the cytoplasm. The subsequent elongation of this vesicle results in the separation of the cytoplasmic components from the rest of the cell to form an autophagosome. The autophagosome then fuses to the lysosome and the contents are enzymatically degraded into amino acids, which can be used for de novo synthesis of proteins. There are three types of autophagy systems: macroautophagy, microautophagy, and chaperone-mediated autophagy; the term autophagy usually indicates macroautophagy unless otherwise specified (Mizushima 2007). The main role of autophagy is to reallocate nutrients from unnecessary processes to more pivotal ones required for survival. In addition, in order to maintain cellular functions and survival, a low-level of constitutive autophagy is also important for maintaining the “quality” of proteins and organelles, as recently reported in various organs, including the liver (Komatsu et al. 2005), brain (Komatsu et al. 2006; Hara et al. 2006), and heart (Nakai et al. 2007). Thus, autophagy functions as a cell-protection mechanism. Paradoxically, autophagy also appears to modulate cell death through self-digestion and degradation of essential cellular constituents (Shimizu et al. 2004; Koike et al. 2008; Shintani and Klionsky 2004).

Molecular mechanisms of induction and regulation of autophagy
In the presence of adequate nutrients, growth factors such as insulin can activate Class I P13 kinase proteins, which in turn signal via the AKT pathway to activate mTOR, which inhibits the activation of autophagy. While autophagy was discovered as early as the 1960s (Deter and De Duve 1967), the molecular mechanisms that explain this phenomenon remained unsolved for years. A quantum leap has been made in the understanding of the molecular mechanisms of autophagy after the identification of a large family of \textit{Atg} genes involved in the autophagy process in yeast (Nakatogawa et al. 2009). These are classified into the following five groups; (i) ATG1 protein kinase and its regulators: mTOR activation leads to inhibition of ATG1. With the lack of adequate nutrients or the presence of mTOR inhibitors, such as rapamycin, ATG1 can recruit ATG11, ATG13 and ATG17 to form a complex that signals induction of autophagy. (ii) ATG12-ATG5 conjugation reactions: the ATG12–ATG5 conjugates form a multimeric complex with ATG16L1. (iii) LC-3 (mammalian homologue of ATG8) conjugation reaction: The ATG12-ATG5 conjugates have an E3-like activity to promote the conjugation of LC3 to the phospholipid, phosphatidylethanolamine. (iv) ATG6 (Beclin1) which interacts with Class III P13 kinase proteins complexes along with ATG14, (v) The ATG9 and ATG2-ATG18 complex, which mediate the disassembly of ATG proteins from mature autophagosomes. Upon induction of autophagy, membranes are induced in the cytosol to form a pre-autophagosome, to which ATG12-ATG5 complexes are localized. During the formation of the autophagosome from the pre-autophagosome, the ATG12-ATG5 complexes are released from those membranes and LC3-II becomes localized on them. Usually, LC3-I (non-lipidated form) is distributed throughout the cytosol and can be localized
to the autophagosomes only when it is lipidated to form LC3-II. ATG7 is a key enzyme involved in both ATG12-ATG5 conjugation and LC3 lipidation, both of which are essential for the elongation of the isolation membranes (Levine and Kroemer 2008). Less well defined than the above processes is the fusion between the autophagosome and the lysosyme and subsequent breakdown of the autophagic vesicle, though the LAMP2 protein plays an important role in this process (Tanaka et al. 2000) (Fig. 1).

**Autophagy in pancreatic β cells**

“Crinophagy”, the process of direct fusion of the secretory granule to the lysosome, has been known for years as an autophagy-related protein degradation mechanism in endocrine cells (Farquhar 1977). It was only recently that autophagy was described in pancreatic β-cells and its potential role in β-cell homeostasis was noted. For example, Li et al reported that autophagosomes are readily detectable in β-cells of Zucker diabetic fatty (ZDF) rats (Li et al. 2006). The first demonstration of the importance of autophagy in pancreatic β-cell homeostasis was reported in a recent study that showed marked up-regulation of autophagy in secretory-deficient Rab3−/− β-cells and the contribution of autophagy to the maintenance of intracellular insulin content through acceleration of the insulin degradation rate in β-cells (Marsh et al. 2007). As a first step toward elucidating the role of autophagy in β-cells, we used electron microscopy to examine the formation of the autophagic vacuole and the in vivo regulatory mechanisms involved in this process. Autophagosome formation was rarely detected in β-cells in C57BL/6 mice under steady-state. Consistent with the notion that
recognition by autophagic vacuoles lacks stringent substrate specificity, which is different from that used by the ubiquitin-proteasome system (Coux et al. 1996), any structure in the cytosol can be a substrate for autophagy. Various types of cellular structures including insulin granules, mitochondria, endoplasmic reticulum membranes were observed in the autophagic vacuoles (Ebato et al. 2008; Jung et al. 2008). In contrast to what is observed in most other organs, such as liver and muscle (Kuma et al. 2004), starvation of C57BL/6J mice for 48 hr does not increase autophagic vacuole formation in β-cells. This is similar to the lack of up-regulation of autophagy in neural tissues of C57BL/6J mice under starvation conditions. These tissues (i.e., neuronal and β-cells) might be selectively protected from intracellular energy deprivation when the animal is starved. Interestingly, active formation of the autophagosome was observed in β-cells when C57BL/6 mice are fed high-fat diets. Similarly, a marked increase in autophagosome formation was observed in db/db mice, compared with db/misty controls. Increased insulin resistance is one of the phenotypes commonly observed in db/db mice and in mice fed a high-fat diet, suggesting a possible involvement of insulin resistance in activation of autophagy.

Role of “basal-autophagy” in β cells

Our study revealed a low-level of constitutive autophagy in pancreatic β-cells under steady-state conditions and its enhancement when mice are fed a high-fat diet (Ebato et al. 2008). What, then, is the physiological role of autophagy? To address this question, we generated mice with selective deficiency in autophagy in β-cells (Atg7<sup>fl/fl</sup>:RIP-Cre mice). At first, the role of “basal autophagy” in β-cells was
investigated by providing the mice a standard diet. The β-cells of these mice showed blockade of LC3-II induction from LC3-I, and marked accumulation of p62, a substrate for autophagic degradation (Ichimura et al. 2008), suggesting efficient suppression of autophagy in β-cells. Furthermore, Atg7<sup>−/−</sup>:RIP-Cre mice developed progressive degeneration of β-cells, characterized by cellular hypertrophy, depletion of insulin immunoreactivity and an increased rate of apoptotic cell death. In addition, large protein aggregates, containing polyubiquitinated proteins and p62, were noted in the cytosol of autophagy-deficient β-cells. Accumulation of protein aggregates positive for polyubiquitinated proteins is a phenotype common to the liver, central nervous system (CNS), and heart after deletion of Atg7 (Komatsu et al. 2005; Komatsu et al. 2006; Hara et al. 2006; Nakai et al. 2007). The finding suggests that the ubiquitin-proteasome system cannot by itself eliminate polyubiquitinated proteins and unnecessary proteins. Polyubiquitinated protein aggregate formation is a typical pathological finding in steatohepatitis (Zatloukal et al. 2002) and neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease (Nakaso et al. 2004). Is it also observed in diabetes? Kaniuk et al. reported age-dependent polyubiquitinated protein aggregate formation in β-cells of ZDF rats and that such aggregate formation is accelerated by hyperglycemia-induced oxidative stress (Kaniuk et al. 2007). Using an in vitro culture system, they reported that autophagy regulates the degradation of polyubiquitinated proteins. Thus, β-cells seem to exhibit reduced autophagic activity, which can result in deterioration of β-cell function under hyperglycemic conditions.

Atg7<sup>−/−</sup>:RIP-Cre mice exhibited impaired glucose tolerance. The glucose tolerance test (GTT) in these mutant mice showed reduced insulin secretion, but no
difference in insulin-induced blood glucose reduction compared with the control. Based on the results, reduction in insulin secretion from β-cells appears to be the primary etiology of impaired glucose tolerance in Atg7ff:RIP-Cre mice. To further clarify this point, detailed analysis were carried out on isolated islets. Glucose-induced insulin release from autophagy-deficient islets was significantly reduced compared with control islets. Given that there was no difference in KCl-induced insulin secretion between the two groups of islets, deficiency of signaling events downstream of KATP channel closure do not appear to be responsible for the defective glucose-stimulated insulin secretion.

Mitophagy and cellular function

Autophagy is implicated in the maintenance of mitochondrial function by facilitating mitochondrial turnover (mitophagy) (Chen and Chan 2009). Consistent with the notion, morphological defects such as distended mitochondria and distorted cristae were frequently observed in Atg7-deficient β cells. Impaired mitochondrial function in mutant β-cells was shown by a reduction in glucose-stimulated ATP production and Ca2+ influx in the mutant β-cells (Ebato et al. 2008; Jung et al. 2008). Thus, reduced basal autophagy can result in deterioration of β-cell function by allowing intracellular accumulation of damaged organelles, particularly dysfunctional mitochondria. Damaged mitochondria can be depolarized and ultimately digested by autophagy. Although the mechanisms by which dysfunctional mitochondria are identified and targeted for autophagy-related elimination is not entirely understood, growing evidence suggests that depolarized, dysfunctional mitochondria can be selectively
recognized by autophagic vacuoles in an Atg32- (Kanki et al. 2009; Okamoto et al. 2009) and/or Pink1-Perkin-dependent manner (Kawajiri et al. 2010; Narendra et al. 2010; Geisler et al. 2010; Vives-Bauza et al. 2010). Wu et al. examined the impact of autophagic deficiency in skeletal muscle by specific deletion of Atg7 in muscle (Wu et al. 2009). The Atg7ff::MCK-Cre mice exhibited defective mitochondrial respiration and accumulation of reactive oxygen species (ROS) in skeletal muscle cells. Thus, autophagy seems to prevent the release of ROS from damaged mitochondria. Wu et al also generated mice with β-cell-specific autophagy deletion (Atg7ff::RIP2-Cre) and again observed increased ROS production in β-cells. In the latter model, administration of an antioxidant ameliorated the physiological impairment in glucose-stimulated insulin secretion but importantly, did not improve autophagy flux, as assessed by LC3-II levels and p62 accumulation (Wu et al. 2009). These results indicate the critical role of oxidative stress in deterioration of β-cell function in the autophagy-deficient β-cells. Given that mitochondrial dysfunction is one of the hallmarks of insulin resistance in the muscle and liver (Cheng et al. 2009), dysfunctional mitophagy may play a key role not only in insulin secretory defects but also in various cellular dysfunctions associated with impaired insulin signaling.

*Induced β-cell autophagy as an adaptive response against increased insulin resistance*

To elucidate the physiological role of induced autophagy in the insulin resistance state, we characterized Atg7ff::RIP-Cre mice fed a high-fat diet for 12 weeks. The mutant mice showed extensive degenerative changes in β-cells associated with
ubiquitinated protein aggregation (Ebato et al. 2008). High-fat diet-fed $Atg^{7/8}:RIP-Cre$ mice exhibited severe glucose intolerance. Furthermore, during the 12-week-high-fat diet, β-cell mass increased by 2-fold in the control mice but such adaptive expansion was severely blunted in the mutant mice. The islets of $Atg^{7/8}:RIP-Cre$ mice fed a high-fat diet were characterized by a small number of Ki67-positive replicating cells and an increased number of caspase-3 positive apoptotic cells. These findings may account for the lack of an adaptive increase in β-cell mass, and suggest that induced autophagy under insulin resistance state is crucial for elimination of protein aggregates, harmful proteins and organelles in β-cells, thereby avoiding cell death and onset of diabetes (Fujitani et al. 2009; Fujitani et al. 2009).

What is the signal that “senses” systemic insulin resistance and in turn activates autophagy in β-cells? In vitro studies indicate that long-chain free fatty acids (FFA), such as oleate and palmitate, stimulate autophagy flux in INS-1β-cells and exposure of β-cells to palmitate enhances the formation of autophagosomes and autolysosomes (Choi et al. 2009; Lupi et al. 2002). In addition, inhibition of autophagosome formation augments FFA-induced β-cell death, suggesting that increased autophagy seems to play a protective role against FFA-induced β-cell death. Based on the observations that FFA can stimulate autophagy in vitro and that in vivo serum FFA levels are elevated in settings of insulin resistance, one can implicate FFA in the activation of autophagy that occurs with insulin resistance (Fig.2).

Beta-cell autophagy and death
While the protective role of autophagy in pancreatic β-cells has been proposed based on loss-of-function studies for Atg genes, autophagy also appears to be involved in certain types of cell death. Masini et al. (Masini et al. 2009) investigated the features of autophagy in β-cells from type 2 diabetic patients. Electron microscopic observation demonstrated the presence of more dead β-cells in islets of diabetics than from non-diabetic controls. The type of cell death observed seems to be apoptosis or autophagy-associated cell death. Further analysis indicated that diabetic β-cells were associated with reduced transcription of LAMP2 and cathepsin B and D. Exposure of non-diabetic islets to high FFA concentrations resulted in marked increase in autophagic vacuole accumulation, together with enhanced β-cell death, which was associated with decreased LAMP2 expression. The above study does not necessarily indicate that the enhanced autophagy is harmful in type 2 diabetics, however, in some circumstances, altered levels or impaired function of autophagy, possibly defects in the process of lysosome fusion and/or proteolytic enzyme activation, may contribute to the reduced β-cell mass by accelerating β-cell death. Another study reported that reduced β-cell mass in pdx1 hetero-knockout mice was associated with increased autophagy in pancreatic islets: Inhibition of autophagy in Becln1 hetero-knockout mice, a model of decreased autophagy, restored β-cell function together with preservation of β-cell mass in pdx1 hetero-knockout mice (Fujimoto et al. 2009). It is surprising that phenotypes caused by pdx1 reduction can be rescued simply by reduction of autophagy, since pdx1 regulates various functions of β-cells, from ontogeny until their differentiated phenotype such as glucose-induced insulin secretion (Kaneto et al. 2007). Furthermore, Becln1/Atg6 was originally isolated as a
Bcl-2-interacting protein and can affect apoptosis beyond its role in autophagic regulation (Liang et al. 1998; Sinha and Levine 2008). These results suggest that accelerated autophagy may contribute to β-cell death under special conditions.

**Autophagy and diabetes**

Loss-of-function experiments have demonstrated that autophagy in β-cells plays a crucial role in the preservation of pancreatic β-cell architecture and function. Importantly, the typical features of type 2 diabetes were phenocopied in *Atg7fl:RIP-Cre* mice: these mice exhibited mildly impaired glucose tolerance due to reduction in glucose-induced insulin secretion when fed a normal diet. The same mice developed diabetes with marked β-cell loss when fed a high-fat diet (Ebato et al. 2008). These observations strongly suggest that reduction in autophagic activity could predispose individuals to type 2 diabetes.

Are there alterations in autophagic activity in diabetes? Electron microscopy analysis has revealed an increased number of autophagosomes in β-cells of diabetic *db/db* mice and ZDF rats, suggestive of up-regulation of autophagy (Li et al. 2006). It is possible that the levels of autophagy induced in *db/db* mice are not sufficient to completely eliminate ubiquitinated proteins and cellular waste produced under insulin-demanding situations. It should be noted, however, that the number of autophagosomes alone does not provide unambiguous evidence of increased autophagic flux. For instance, increased numbers of autophagosome in *db/db* mice may indicate decreased flux caused by a defective autophagic pathway, such as
impaired autophagosome/lysosome fusion, or activation of lysosomal enzymes (Levine and Kroemer 2008; Tanaka et al. 2000). Accumulation of p62, a substrate for autophagy (Ichimura et al. 2008), in β-cells of db/db mice could suggest defects in the autophagic machinery. Alteration of autophagic activity in diabetic animal models therefore needs to be carefully assessed in future experiments. As discussed in a previous study (Masini et al. 2009), a next question would be whether altered levels of β-cell autophagy play a pathogenic role in type 2 diabetes. To address this, suitable non-invasive biomarkers for monitoring the levels of intracellular autophagy in pre-diabetic subjects need to be established. Various stressors such as ER stress and oxidative stress play a role in the regulation of β-cell function and survival accompanied by progression of diabetes. Growing evidence suggests that ER stress and oxidative stress can affect cellular functions through the modulation of autophagic pathways (Ogata et al. 2006; Bernales et al. 2006; Yorimitsu et al. 2006). It is plausible that misfolded proteins produced in β-cells are degraded by the ER-associated protein degradation (ERAD) system (Metzger et al. 2008) in cooperation with autophagic degradation, both of which might be activated by ER stress sensors. To examine in vivo the direct link between ER stress and autophagy, we are currently investigating the activation status of autophagy in the Akita diabetic mouse, a mice model where ER stress induced by misfolded insulin is the primary cause of β-cell dysfunction (Ron 2002).

Concluding remarks
Pancreatic β-cells play a central role in glucose homeostasis in mammals. Although large scale protein synthesis and degradation occur in β-cells, the mechanism underlying dynamic protein turnover in β-cells remains largely unknown. In β-cells, autophagy is important for the turnover of protein aggregates and organelles at basal levels under normal conditions and is up-regulated in response to increased insulin resistance. Based on the analysis of β-cell-specific Atg7-deficient mice, autophagy protects β-cells against cell death and allows compensatory cell expansion in response to metabolic demands. Given that autophagy is a cellular response to multiple factors that influence cellular homeostasis, including food constituents, glucoregulatory hormones and therapeutic drugs, dysregulation of autophagic pathways in various organs is potentially linked to metabolic disorders such as diabetes mellitus. Elucidation of the role of autophagy in mediating either cell survival or cell death during the progression to diabetes, as well as the design of techniques that allow manipulation of autophagy, could have a significant impact on the therapeutic approaches to diabetes. Such clinical utility will likely depend, at least in part, on efforts to assess whether autophagy contributes to development and progression of diabetes mellitus in patients with the disease, in addition to experimental animals. Such clinical studies are an important challenge for the future.

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FIGURE LEGENDS

Figure 1. Intracellular pathways regulating macroautophagy
Macroautophagy includes distinct stages: vesicle nucleation, vesicle elongation and completion, fusion of the double-membrane autophagosome with the lysosome to form an autolysosome, and lysis of the autophagosome inner membrane and breakdown of its contents inside the autolysosome. Atg proteins form different complexes that function in distinct stages of autophagy. Shown here are the complexes that have been identified in mammalian cells, with the exception of Atg13 and Atg17 that have only been identified in yeast.

Figure 2. Role of induced β-cell autophagy in insulin resistance. Exposure of β-cells to various stresses caused by peripheral insulin resistance may induce ubiquitination of proteins and storage of damaged organelles into cytoplasmic aggregates of pancreatic β-cells. The ubiquitinated proteins and damaged organelles are effectively cleared by induced β-cell autophagy. Autophagy can also act as a defense mechanism against various types of cellular damage in β-cells. This process is especially essential for the compensatory increase in β-cell mass in the presence of insulin resistance.
REFERENCES


27. Nakaso K, Yoshimoto Y, Nakano T, Takehashi T, Fukuhara Y, Yasui K,


40. Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C,


46. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S,


Fig. 2

Insulin resistance

FFA ↑ ???

Diabetes

Autophagic activity ↑

No diabetes

Degradation of unnecessary protein damaged mitochondria

Poor β-cell compensation

Cell death

Cell proliferation

Good β-cell compensation

Cell proliferation

Cell death