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The double-edged effect of autophagy in pancreatic beta cells and diabetes

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Autophagy is an intracellular catabolic system, which enables cells to capture cytoplasmic components for degradation within lysosomes. Autophagy is involved in development, differentiation and tissue remodeling in various organisms, and is also implicated in certain diseases. Recent studies demonstrate that autophagy is necessary to maintain architecture and function of pancreatic beta cells. Altered autophagy is also involved in pancreatic beta cell death. Whether autophagy plays a protective or harmful role in diabetes is still not clear. In this review, we will summarize the current knowledge about the role of autophagy in pancreatic beta cell and diabetes.

Introduction

Macroautophagy is a biological process in which double-membrane cytosolic vesicles called autophagosomes sequester cytoplasm, subsequently delivering it to lysosomes where the engulfed cellular components are degraded. The major types of autophagy are macroautophagy, microautophagy and chaperone-mediated autophagy.[1] Macroautophagy is the most studied mechanism of autophagy. Here, we will focus on macroautophagy, which is hereafter called autophagy. On the one hand, autophagy is markedly upregulated during conditions of nutrient or growth factor deficiency to maintain the normal functioning of cellular structures and provide energy for cell survival.[2] On the other hand, autophagy is thought to be a cell-fate decision maker, and can act as a trigger of apoptosis referred to as autophagy-dependent cell death. A blockade in the autophagic flux leads to apoptosis in glioma cells.[3] CD4/CXCR4-expressing T cells that interact with cells expressing HIV-1-encoded envelope glycoproteins first manifest autophagy and then apoptosis. In this model, depletion of Beclin 1 or ATG7 inhibits apoptosis.[4] Autophagy may also represent a form of programmed cell death called autophagic cell death or type 2 programmed cell death. Autophagic cell death is characterized by the accumulation of autophagic vesicles, which is distinct from type 1 programmed cell death or apoptosis.[5] Autophagy is also implicated in various diseases such as cancer, muscular disorder and neurodegeneration.[6] In this review, we will summarize the current knowledge about the connections between autophagy and diabetes.

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Autophagy and Degradation of Cellular Components

Pancreatic beta cells have the main functions of insulin synthesis and secretion, which play a key role in glucose homeostasis. Glucose is a well-known stimulus of proinsulin biosynthesis. Insulin biosynthesis approaches 50% of total protein synthesis in stimulated beta cells,[7] and the endoplasmic reticulum (ER) is responsible for the synthesis of almost all secreted proteins. These proteins are correctly folded and assembled by chaperones in the ER lumen. When the glucose concentration increases, the insulin demand also is markedly increased. As a result, the chaperones become overloaded and the ER fails to fold and export newly synthesized proteins, leading to ER stress.[8] ER stress can result in the accumulation of misfolded proteins, and is necessary for lipid-induced apoptosis in beta cells.[9] In addition to ER stress, pancreatic beta cells are prone to oxidative stress, due to the fact that antioxidants such as superoxide dismutase (SOD), glutathione peroxidase and catalase are present at low levels in beta cells. Lenzen et al. studied gene expression of SOD, catalase and glutathione peroxidase in pancreatic islets and for comparison in various other mouse tissues using a sensitive northern blot hybridization technique.[10] Gene expression of the antioxidant enzymes was usually in the range of ±50% of that in the liver. They found that in pancreatic islets gene expression was substantially lower. The levels of the cytoplasmic Cu/Zn SOD and the mitochondrial Mn SOD gene expression were in the range of 30–40% of those in the liver. Glutathione peroxidase gene expression was 15% and catalase gene expression was not at all detectable in pancreatic islets.[10] Oxidative stress can also lead to the accumulation of misfolded proteins.

Protein degradation is mediated by two major systems; the ubiquitin proteasome system and autophagy. The ubiquitin proteasome system is a major degradation system for short-lived proteins, whereas autophagy is mainly responsible for the degradation of long-lived proteins and other cellular contents. Autophagy is activated when the ubiquitin proteasome system is inhibited, which suggests the two systems are functionally linked.[11] Ubiquitinated-protein aggregates increased in INS-1

cells (a mouse insulinoma cell line) in response to high glucose. The formation and clearance of ubiquitinated-protein aggregates was regulated by autophagy. Inhibition of the proteasome did not affect the clearance of ubiquitinated proteins in INS-1 cells. In contrast, an increase in ubiquitinated-protein aggregates was observed in cells treated with 3-methyladenine (3-MA), an autophagy inhibitor; the level of aggregates was higher than that seen in cells grown in high glucose alone.[12] Similarly, deletion of autophagy-related (Atg) gene 7 (*Atg7*) also led to the accumulation of ubiquitinated-protein aggregates.[13] These results demonstrate that autophagy plays a critical role in the degradation of cellular components including ubiquitinated proteins that are associated with diabetes.

Autophagy is Indispensable for the Maintenance of Normal Beta Cell Architecture

In *Atg7*-deficient mice, multiple cyst-like structures were observed in beta cells via haematoxylin and eosin (H&E) staining. Toluidine-blue staining of thin-sliced sections showed that mutant islets contained enlarged "balloon-like" cells with a pale-stained cytoplasm located at the islet periphery, which corresponded to the cyst-like structures in H&E sections. Electron microscopy analysis indicated that the "balloon-like" cells were most likely degenerating beta cells, on the basis of the observation that they were relatively rich in mitochondria and always associated with a small number of insulin vesicles.[14] More ultrastructural abnormalities such as swelling of mitochondria and cisternal distension of the rough ER and the Golgi complex were also observed in *Atg7*-deficient beta cells.[13] All these results indicate that autophagy is indispensable for the maintenance of normal beta cell architecture.

Autophagy, β-Granules and Insulin Secretion

Endocrine cells are continually regulating the balance between hormone biosynthesis, secretion and intracellular degradation to ensure that cellular hormone stores are maintained at optimal levels. Insulin is stored in β-granules in pancreatic beta cells. Normally, a beta cell contains a relatively constant number of β-granules, but β-granules are continually turned over, with an estimated half-life of 3–5 days.[15] Under normal circumstances, older β-granules in the beta cell's storage pool are eventually degraded via crinophagy, whereby a β-granule fuses directly with a large lysosomal-related vacuolar compartment resulting in degradation of the β-granule lumenal content.[16-19] However, crinophagy alone is not sufficient to maintain optimal insulin storage when insulin secretion is dysfunctional.

In the *Rab3A*$^{-/-}$ null mouse (insulin secretion-deficient animal model) islet beta cells, glucose-regulated insulin secretion was significantly deficient, approximately 70% less compared with control islets. In contrast, control of insulin biosynthesis was normal in *Rab3A*$^{-/-}$ islets and preproinsulin mRNA levels and glucose-induced proinsulin biosynthesis did not change compared with *Rab3A*$^{+/+}$ islet cells. Furthermore, proinsulin conversion to insulin was normal in *Rab3A*$^{-/-}$ islets. Despite this net overproduction of insulin (i.e., the internal amount was increased relative to the amount normally secreted), the insulin content did not markedly increase in *Rab3A*$^{-/-}$ mouse islets. This means that beta cells have to degrade the excess insulin to maintain optimal insulin storage. The enhanced degradation of intracellular insulin was mediated by increased crinophagy and autophagy. Lysosome- associated membrane protein 2 (*LAMP-2*) gene expression was reduced approximately 80% in *Rab3A*$^{-/-}$ islets compared with control islets.[20] LAMP-2 is a negative regulator of autophagy.[21] Thus, reduced expression of *LAMP-2*, which correlates with the increased autophagy, suggests that autophagy plays a pivotal role in insulin degradation.[20] In this case, autophagy appears to be involved in maintaining the normal intracellular insulin content by accelerating the insulin degradation rate in beta cells.

Autophagy is also crucial for insulin secretion. In *Atg7*-deficient beta cells, electron microscopy revealed that some beta cells contained remarkably reduced numbers of insulin granules. Correspondingly, basal insulin secretion from primary islets of *Atg7*-deficient mice was significantly decreased. High glucose-stimulated insulin secretion was also markedly reduced in *Atg7*-deficient beta cells compared to control mice.[13]

Inductive Beta Cell Autophagy as an Adaptive Response against Increased Insulin Resistance

Glucotoxic and dyslipidemic states are part of the metabolic syndrome associated with type 2 diabetes. Glucotoxicity and lipotoxicity contribute to the reduction of beta cell mass in type 2 diabetes. Our recent unpublished study demonstrated that after INS-1beta cells were treated with high-glucose for 24 h and 48 h, the apoptotic cells accounted for 19.45% and 38.38%, respectively (investigated using flow cytometry), which was significantly higher than the control (4.95%). Autophagy was also markedly increased after treatment with high glucose. When treated with 3-MA, an autophagy inhibitor, apoptosis was significantly increased compared with cells treated with high glucose only. These results indicate that autophagy plays a protective role in high-glucose induced INS-1beta cell apoptosis.

Autophagy is activated as an adaptive response, providing cells with a safety mechanism to eliminate damaged mitochondria and/or unnecessary proteins and to avoid apoptosis under insulin-resistant states. Fujitani Y et al.[22] reported that compared with *Atg7*$^{f/f}$ mice fed with a high-fat diet for 12 weeks, *Atg7*$^{f/f}$*:Cre* mice (*Atg7*-deficient mice) had significantly higher nonfasting glucose levels and severely impaired glucose tolerance. Morphometric analysis showed a failure of compensatory hyperplasia of beta cells in *Atg7*$^{f/f}$*:Cre* mice, accompanied by a smaller number of Ki67^{+} replicating cells (i.e., cells that are associated with cellular proliferation) and accelerated accumulation of caspase-3^{+} apoptotic cells compared with *Atg7*$^{f/f}$ mice. Degenerative changes in beta cells of *Atg7*$^{f/f}$*:Cre* mice become more evident when these mice were fed with a high-fat diet; the number of degenerated vacuoles and p62 accumulation was further increased.[14,22] Large ubiquitinated-protein aggregates were

observed in the pancreas of Zucker diabetic fatty rats (an obese, type 2 diabetes model).[12] The p62 protein was a ubiquitin-binding scaffold protein that colocalized with ubiquitinated protein aggregates in many neurodegenerative diseases. The protein was itself degraded by autophagy and may serve to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome.[23]

Serum free fatty acids (FFA) are a candidate mediator of these events and the levels are increased in insulin-resistant states. FFA consist of saturated and monounsaturated fatty acids, which have distinct effects on beta cells. The saturated palmitic acid reduces the proliferative capacity of beta cells and induces beta cell death. Palmitoleic acid, a monounsaturated fatty acid with identical carbon chain length, counteracts the toxic effects of palmitic acid and promotes β-cell proliferation. Palmitic acid induces cell death mainly through apoptosis, characterized by cytochrome *c* release, caspase-3 activation and DNA fragmentation.[24]

The concept that autophagy is regulated by FFA is also supported by a recent in vitro study in which FFA-induced activation of autophagy in beta cells is an adaptive response for beta cell survival under increased insulin resistance. Maedler et al. demonstrated that palmitate (PA) was able to elicit the apoptotic death of INS-1beta cells and the cytotoxic effect of PA was enhanced in the presence of higher concentrations of glucose.[24] PA activated the entire process of autophagy; double-layered curved structures (presumably corresponding to phagophores), double-membrane autophagosomes and single-membrane autolysosomes were simultaneously observed in PA-treated INS-1 cells. ER stress and class III phosphatidylinositol 3-kinase were involved in PA-induced activation of autophagy. PA-induced INS-1 cell death was reduced when autophagy was enhanced. Conversely, PA-induced INS-1 cell death could be augmented when the autophagy system was blocked at the stage of autophagosome formation, during fusion between autophagosomes and lysosomes, or at the proteolytic degradation stage in the lysosome.[25]

Autophagic Cell Death Contributes to Loss of Pancreatic Beta Cell Mass in Diabetes

In type 1 diabetes there is a close-to-complete loss of the pancreatic beta cells by autoimmune destruction and, hence, endogenous insulin production is markedly insufficient, so that insulin must be provided via exogenous injection or islet/pancreatic transplantation. Type 2 diabetes is also acknowledged to be a disease of insulin insufficiency. The beta cell mass and/or acquired beta cell dysfunction can no longer adequately cope with the insulin demand in an insulin-resistant setting. The pathophysiology underlying type 2 diabetes is progressive pancreatic beta cell failure with consequently reduced insulin secretion and insulin resistance in peripheral tissues. Whereas beta cell apoptosis plays a major role in reducing beta cell mass in diabetes, autophagic cell death can also contribute to loss of beta cell mass (**Fig. 1**).

Masini et al.[26] studied endocrine cells of human pancreatic samples (from patients with type 2 diabetes and nondiabetic controls) by electron microscopy. Dead beta cells accounted for 2.24 ± 0.53% and 0.66 ± 0.52% of beta cells in diabetic and control specimens, respectively. Dead beta cells with signs of apoptotic death made up 1.06 ± 0.43% of beta cells in diabetic islets and 0.30 ± 0.32% in control islets ($p < 0.05$). Dead beta cells with massive vacuole overload and no major chromatin condensation (signs of autophagy-associated cell death) accounted for 1.18 ± 0.54% of beta cells in diabetic islets and 0.36 ± 0.26% in control islets. Unchanged gene expression of *beclin 1* and *Atg1* and reduced transcription of *LAMP-2* and the genes encoding cathepsin B and D was observed in type 2 diabetic islets. The beta cell death increased when nondiabetic islets were exposed to nonesterified fatty acid (NEFA), and the number of dead beta cells with massive vacuole overload was also augmented. In addition, NEFA-exposed cells showed reduced *LAMP-2* expression. These results demonstrate that autophagy may also contribute to loss of beta cell mass.

The possibility that activation of the autophagy may increase beta cell death is also addressed in a recent in vitro and in vivo study. Fujimoto et al.[28] reported that knockdown (KD)-expression of pancreatic duodenal homeobox 1(Pdx1) in MIN6 cells (a mouse insulinoma cell line) could lead to cell death. Pdx1 is a transcription factor that is essential for normal pancreatic beta cell function and survival. Complete deficiency of Pdx1 is associated with pancreatic agenesis and partial deficiency leads to reduced insulin secretion and beta cell mass.[27] Autophagy was activated in Pdx1-reduced MIN6 cells. The autophagy inhibitor 3-MA prevented Pdx1 KD-induced MIN6 cell death, but 3-MA-treated Pdx1 KD MIN6 cells finally died by caspase-3-dependent cell death. Thus, autophagic cell death appears to occur earlier than apoptosis and inhibition of autophagy delays Pdx1 KD-induced MIN6 cell death.

Pdx1$^{+/-}$ mice were crossed to *beclin 1*$^{+/-}$ mice to inhibit autophagy. Autophagy was reduced but not completely inhibited in *Pdx1*$^{+/-}$ *beclin 1*$^{+/-}$ mice islets, which suggested persistence of basal autophagy in *Pdx1*$^{+/-}$ *beclin 1*$^{+/-}$ beta cells. After 1 week on a high fat diet, compared with *Pdx1*$^{+/-}$ mice, *Pdx1*$^{+/-}$ *beclin 1*$^{+/-}$ mice showed significantly lower blood glucose concentrations, accompanied by significant increases in both fasting and 30-min insulin levels after glucose administration. *Pdx1*$^{+/-}$ *beclin 1*$^{+/-}$ mice displayed inhibition of autophagy, reduction of caspase-3-dependent apoptosis and augmentation of beta cell proliferation compared with *Pdx1*$^{+/-}$ mice.[28]

Beclin 1 and Atg5 are key mediators of autophagosome formation. Beclin 1 is the mammalian orthologue of the yeast Atg6/Vps30 protein and a regulator of the class III phosphatidylinositol 3-kinase complex involved in autophagosome formation.[29] Reducing Beclin 1 expression in Pdx1 KD MIN6 cells by 30% and 50% decreased MIN6 cell death. However, MIN6 cell death increased when Beclin 1 expression was reduced by 90%. These results indicate that a minimum basal level of autophagy is required for MIN6 cell survival.[28]

These latter findings imply a crosstalk between autophagy and apoptosis in the beta cell, as demonstrated in other cellular systems. Beclin 1 is originally identified as an interaction partner of Bcl-2, an anti-apoptotic protein. This Bcl-2-Beclin

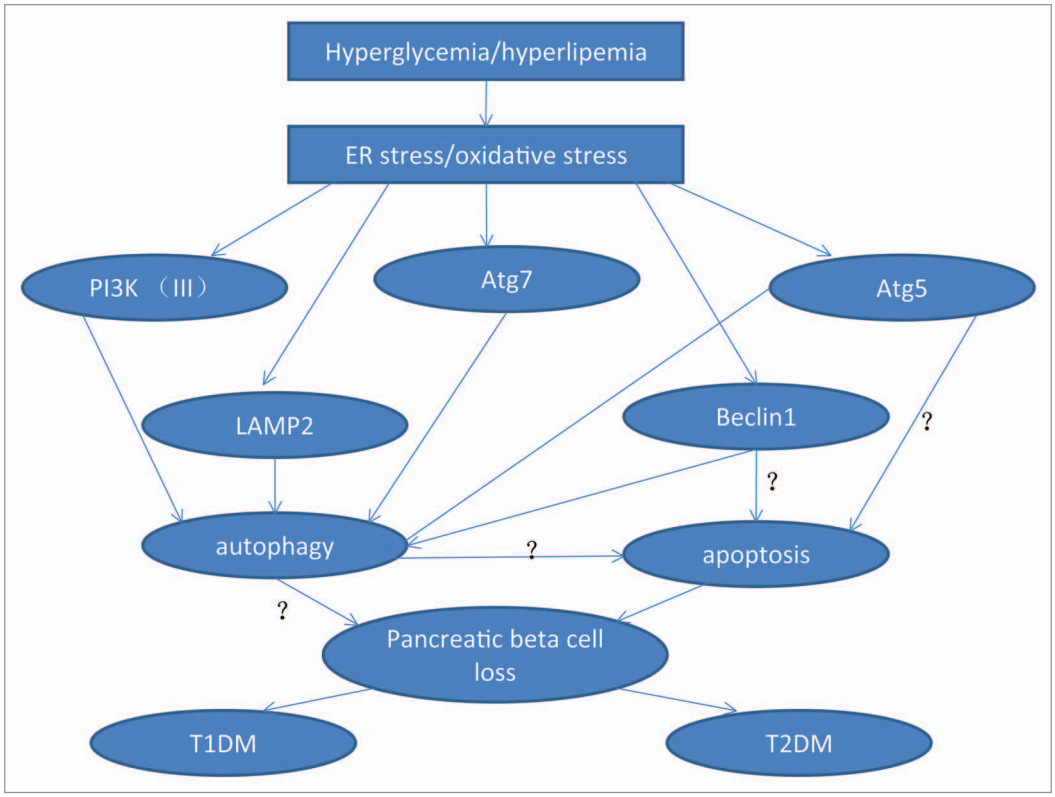

Figure 1. Autophagy, apoptosis and diabetes. ER and oxidative stress triggered by hyperglycemia or hyperlipaemia induce autophagy in pancreatic beta cells. Phosphatidylinositol 3-kinase (PI3K) III, expression of Atg7, Atg5, Beclin 1 and LAMP-2 play a crucial role in regulating autophagy. Whether autophagy plays a protective or harmful role in diabetes is still not clear, and many questions need to be answered. For instance, does autophagy-dependent apoptosis occur in pancreatic beta cells? Is there a cross-talk between autophagy and apoptosis in the beta cell through cleavage products of Beclin 1 and Atg5, as demonstrated in other cellular systems? These and other questions await the results of further research.

1 interaction is mediated through a BH3 domain in Beclin 1. Endogenous Beclin 1 localizes to the two major sites in the cell where Bcl-2 is found, the mitochondria and the endoplasmic reticulum, but only ER-targeted Bcl-2 effectively suppresses autophagy. The ability of normal cells to increase their autophagic activity in response to starvation seems to be regulated by the dissociation of the Bcl-2-Beclin 1 complex. This indicates Bcl-2 is not only an anti-apoptotic protein but also an anti-autophagic protein.[30]

Yousefi et al.[31]demonstrated that an elevated Atg5 level sensitized tumor cells to anticancer drug treatment both in vitro and in vivo. Increased Atg5 levels were associated with increased caspase-3 activity, as well as with increased apoptotic morphology and phosphatidylinositol redistribution within the plasma membrane, suggesting that the type of death was apoptosis. Calpain-mediated Atg5 cleavage resulted in an amino-terminal cleavage product with a relative molecular mass of 24 kDa. Truncated Atg5 translocated from the cytosol to mitochondria, associated with the anti-apoptotic molecule Bcl-x_L and triggers cytochrome *c* release and caspase activation. Wirawan et al.[32] reported that after Beclin 1 was cleaved by caspases, the N-terminal (Beclin 1-N) and C-terminal (Beclin 1-C) cleavage products failed to induce autophagy. Instead, Beclin 1-C translocated from the cytosol to mitochondria and amplified apoptosis.

Conclusion

In recent articles, signs of altered autophagy were observed in the beta cells of the Zucker diabetic fatty rat and in a beta cell line following prolonged exposure to high glucose. In addition, autophagy was essential for the maintenance of normal islet architecture and played a crucial role in maintaining the intracellular insulin content by accelerating the insulin degradation rate in beta cells of the *Rab3A*$^{-/-}$ null mouse. Further demonstration of the importance of normal autophagy for the preservation of beta cells has been recently provided; impaired glucose tolerance and lower insulin levels in beta cell-specific *Atg7* knockout mice had been reported. These phenotypes were accompanied by vacuolar changes, mitochondrial swelling and ER distension in beta cells. Nonetheless, altered autophagy is also associated with loss of beta cell mass in diabetes.

Thus, it is still unclear as to whether autophagy plays a protective or harmful role in diabetes. The regulatory pathway controlling autophagy in pancreatic beta cells is not clear. Recent articles are mostly focused on the effect of autophagy in type 2 diabetes. The potential connection between autophagy and type 1 diabetes has not been mentioned yet.

Masini et al.[26] reported that the anti-diabetic drug metformin could normalize *LAMP-2* transcription and caused a decrease in the number ofdead beta cells with vacuole engulfment. Further

research is needed to determine whether autophagy can be precisely modulated to prevent the appearance or alleviate the progress of diabetes. Thus, autophagy may be a new target for the prevention or treatment of diabetes.

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