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Autophagy and VMP1 Expression Are Early Cellular Events in Experimental Diabetes

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Key Words
Autophagy · Diabetes · Streptozotocin · Pancreas beta cell

Abstract
Background/Aims: We have described VMP1 as a new protein which expression triggers autophagy in mammalian cells. Here we show that experimental diabetes activates VMP1 expression and autophagy in pancreas beta cells as a direct response to streptozotocin (STZ). ***Methods:*** Male Wistar rats were treated with 65 mg/kg STZ and pancreas islets from untreated rats were incubated with 1 mM STZ. ***Results:*** RT-PCR analysis shows early VMP1 induction after STZ treatment. In situ hybridization reveals VMP1 mRNA in islet beta cells. Electron microscopy shows chromatin aggregation and autophagy morphology that was confirmed by LC3 expression and LC3-VMP1 co-localization. Apoptotic cell death and the reduction of beta cell pool are evident after 24 h treatment, while VMP1 is still expressed in the remaining cells. VMP1-Beclin1 colocalization in pancreas tissue from STZ-treated rats suggests that VMP1-Beclin1 interaction is involved in the autophagic process activation during experimental diabetes. Results were confirmed using pancreas islets, showing VMP1 expression and autophagy in beta cells as a direct effect of STZ treatment. ***Conclusion:*** Pancreas beta cells trigger VMP1 expression and autophagy during the early cellular events in response to experimental diabetes.

Introduction

Diabetes mellitus (DM) is the most prevalent human endocrine disease, and it results from loss and/or dysfunction of insulin-secreting beta cells in pancreatic islets. Type 1 DM is a consequence of a massive beta cell destruction, and it is usually characterized by the presence of autoimmunity activity and markers (anti-GAD, anti-islet cell, or anti-insulin antibodies), which reflects the autoaggressive process that leads to beta cell destruction. Type 2 DM is the most common form of the disease. It results from a progressive decline of beta cell function in the context of insulin resistance. Patients with type 2 DM have insulin resistance and relative, rather than absolute, insulin deficiency. STZ is an agent used widely to cause experimental diabetes because of its ability to selectively target and destroy insulin-producing pancreatic islet beta cells [1]. The glucose moiety of STZ allows preferential uptake of the toxin into beta cells via the glucose transporter, GLUT-2 [2]. STZ is an alkylating agent that causes breakage of DNA strands. This leads to the activation of poly(ADPribose) synthetase and a rapid and lethal depletion of NAD in beta cells [3]. The effect of STZ in beta cells has also been ascribed to the production of nitric oxide [4, 5] and hydroxyl radicals or ROS [6, 7]. However, molecular mechanisms activated by beta cells in response to STZ-induced injury are not fully elucidated.

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Autophagy is an evolutionarily preserved degradation process of cytoplasmic cellular constituents, which serves as a survival mechanism in starving cells [8, 9]. This catabolic process is involved in the turnover of long-lived proteins and other cellular macromolecules, and might play a protective role in the development, aging, and defense against intracellular pathogens [10, 11]. Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called autophagosomes, which eventually acquire lysosomal-like features [12, 13]. By morphological studies, autophagy has been linked to a variety of pathological processes such as neurodegenerative diseases and tumorigenesis, which highlight its biological and medical importance [14, 15]. Autophagy has been proposed to participate in the control of insulin content in secretory-deficient pancreas beta cells [16]. Moreover, autophagy participates in dysfunctional mitochondria as well as the elimination of ubiquitinated protein aggregates in beta cells during oxidative stress [17, 18]. Recently we have described a transmembrane protein which expression triggers autophagy in mammalian cells interacting with Beclin1 to start the autophagosome formation [19]. This new autophagy-related protein named VMP1 is not constitutively expressed in pancreas but is highly activated in acinar cells undergoing autophagy during acute pancreatitis [20, 21]. In this study, we investigate whether beta cells activate the autophagic process in response to experimental diabetes. We show that STZ treatment induces VMP1 expression and autophagy in pancreas beta cells as early cellular events during experimental diabetes. We also demonstrate that VMP1 expression and autophagy are activated in pancreas islets as a direct response to STZ treatment. Furthermore, our results suggest that VMP1-Beclin1 interaction is involved in the autophagic process activated in beta cell in response to experimental diabetes.

Material and Methods

Animals

Male Wistar rats weighing 150–200 g were used. Animals were housed with free access to food and water. Experiments were performed according to the standard ethical and legal guidelines of the Administración Nacional de Medicamentos, Alimentos y Tecnología Medica and the University of Buenos Aires.

Isolated Islets

Pancreatic islets were isolated from pancreas of adult male Wistar rats treated with collagenase (Sigma) according to the method of Lacy and Kostianovsky [22]. Isolated islets were cultured in RPMI medium (Sigma) supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) [23].

STZ Treatment

Experimental diabetes was induced by a single intraperitoneal injection of 65 mg/kg body weight of streptozotocin (Sigma, St. Louis, Mo., USA) dissolved in a citrate buffer (0.1 mol/l, pH 4.5). Controls were injected with citrate buffer. Animals were killed by decapitation at different times after diabetes induction. Pancreases were removed and immediately trimmed free of fat in cold phosphate-buffered saline (PBS). For in vitro STZ treatment, 15 isolated islets per tube were exposed 45 min to 1 mM STZ in citrate buffer at 37°C and then incubated during 4 h at 37°C. Control islets were incubated with the same volume of citrate buffer.

TUNEL Assay

Duplicate 5-μm-thick, formalin-fixed and paraffin-embedded tissues were used. Apoptosis was detected by TUNEL (Tdt-mediated dUTP-biotin nick-end labeling). Sections for TUNEL reactions were processed as described for the In Situ Cell Death Detection Kit (Promega).

In situ Hybridization

The rat VMP1 complementary RNA (cRNA) probe was transcribed from the 1,858-bp *Not-Eco*RI rat VMP1 cDNA inserted into plasmid pT7T3D (Amersham Pharmacia Biotech). Templates were either linearized with *Eco*RI to be used for in vitro antisense transcription using T3 RNA polymerase, or with *Not*I followed by the sense cRNA transcription using T7 RNA polymerase. After linearization, cRNA probes were labeled with DIG-UTP (Roche Molecular Biochemicals). The in situ hybridization method was as described by Komminoth et al. [24] with modifications. Duplicate 5-μm-thick, formalin-fixed and paraffin-embedded tissue sections placed on Superfrost Plus-treated slides (Fisher Scientific) were used. As a negative control, the sections were treated with RNAse A prior to prehybridization, and a second negative control was performed using the rat VMP1 sense cRNA as probe. The hybridization signal was detected according to the instructions of the Dig Nucleic Acid Detection Kit (Roche Molecular Biochemicals).

Histological Studies

Tissues were fixed in 1% glutaraldehyde-0.1 M phosphate buffer and postfixed in osmium tetroxide in the same buffer. After immersion in uranyl acetate, the samples were dehydrated in a graded concentration of ethanol and cleared with acetone. They were embedded in Embed 812 (Electron Microscopy Scientific). 1-μm-thick sections were stained with 1% toluidine blue in borax.

Immunohistochemistry and Immunofluorescence

Sections were immunostained using an alkaline phosphatase or Alexa fluorocromes (Molecular Probes, Invitrogen) indirect labeling technique. Duplicate 5-μm-thick, formalin-fixed and paraffin-embedded tissues were used. Polyclonal rabbit antiserum to VMP1 was used at 1:100 dilution. Polyclonal goat anti-LC3, anti-Beclin1 and rabbit anti-insulin polyclonal (Santa Cruz Biotech) antibodies were used according to the manufacturer's instructions. Donkey anti-rabbit Alexa Fluor 488 and donkey

anti-goat Alexa Fluor 594 (Molecular Probes) and mouse anti-rabbit IgG-AP (Santa Cruz Biotech) antibodies were used for immunostaining. Alkaline phosphatase staining was visualized by incubation with Fast Red TR (Sigma).

Transmission Electron Microscopy

Cells were fixed without being brought into suspension and processed for transmission electron microscopy by standard procedures. Grids were examined under a Carl Zeiss C-10 electron microscope (LANAIS-MIE, UBA).

RT-PCR

RT-PCR was performed using standard procedures. Total RNA was extracted from pancreas using Trizol Reagent (Invitrogen-Gibco). RNA was reversely transcribed using ImPromII reverse transcriptase (Promega) as recommended by the manufacturer, with specific primers. The template was amplified by PCR under the following conditions: denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 59°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. Primer pairs used in this study were: VMP1 sense 5′-TATGCCAAACGAATCCAGCAG-3′, and VMP1 antisense 5′-GAGGGTGTGCAATGCATCATA-3′. GAPDH mRNA was used as an internal control.

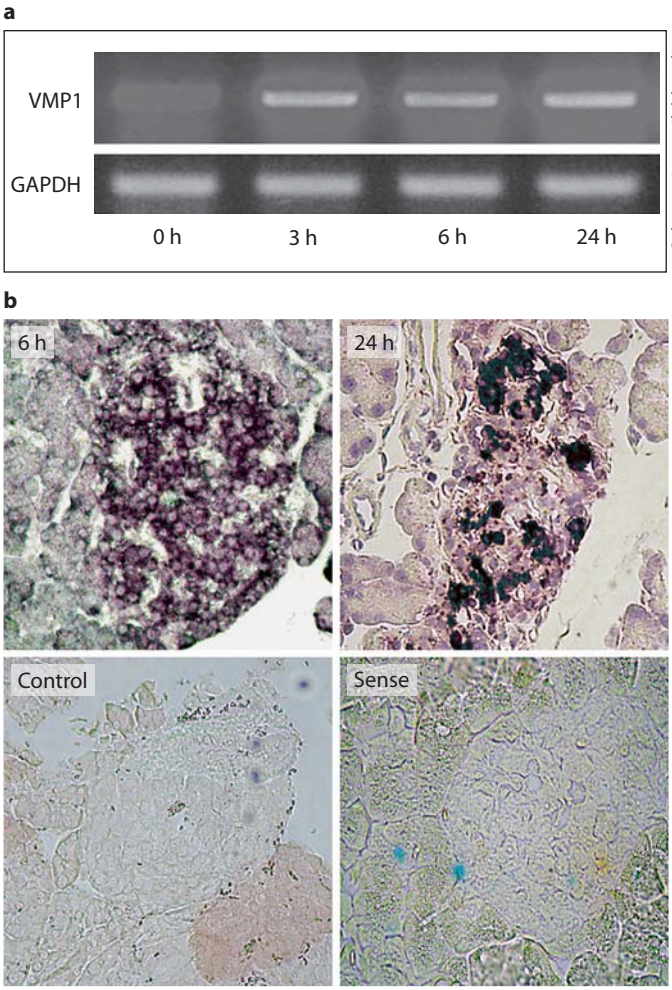

Fig. 1. VMP1 gene expression is activated in pancreas by STZ treatment. Male Wistar rats were treated with single dose of STZ (65 mg/kg body weight) and pancreas removed at different time intervals. **a** RT-PCR analysis of STZ-treated rat pancreas in a time course scheme. Results are representative of 3 independent experiments. **b** In situ hybridization of VMP1 transcript. Pancreas tissue slides from control and STZ-treated rats were hybridized with the digoxigenin-labeled antisense VMP1-RNA probe. Positive VMP1-RNA stain is observed in central islet cells, while no signal is detected in exocrine cells. No signal is present neither in control pancreas nor in sense probe as control. Results are representative of three independent experiments.

Results

STZ treatment in rats induces a significant increase in serum glucose during the first 24 h of administration. Glycemia was 26.4 ± 1.2 mmol/l in diabetic animals versus 6.3 ± 0.6 mmol/l in controls ($p < 0.001$). In order to know if VMP1 gene expression is activated by STZ treatment, we investigated VMP1 mRNA in pancreas by RT-PCR analysis. Figure 1a shows the time course of VMP1 mRNA expression in pancreas. There is no detectable basal expression but it is activated 3 h after STZ administration; it becomes maximal 6 h after and remains activated after 24 h. To identify cell types that express the VMP1 transcript, we performed in situ hybridization assay. Pancreas tissue slides from control and STZ-treated rats were hybridized with the digoxigenin-labeled antisense VMP1-RNA probe. Figure 1b shows VMP1 transcript signal 6 and 24 h after STZ administration. VMP1 mRNA expression is observed in central islet cells, while no signal is detected in exocrine pancreas, showing that VMP1 expression in response to STZ is restricted to endocrine pancreas. Pancreases from untreated rats show no signal of the transcript. Hybridization with a digoxigenin-labeled sense RNA probe was performed as control. We then analyzed insulin and VMP1 protein expression by immunohistochemistry. Impaired number of insulin immunoreactivity cells is evident during STZ treatment (fig. 2a). VMP1-positive cells are observed 6 h after STZ in central islet cells (fig. 2b). After 24 h, the number of insulin immunoassayed cells is reduced and apoptosis is evident by TUNEL assay (fig. 2c). Nevertheless, VMP1 is still expressed in remaining beta cells (fig. 2b).

VMP1 is an autophagosomal membrane protein which expression triggers autophagy in mammalian cells. VMP1 interacts with Beclin1, a mammalian autophagy initiator

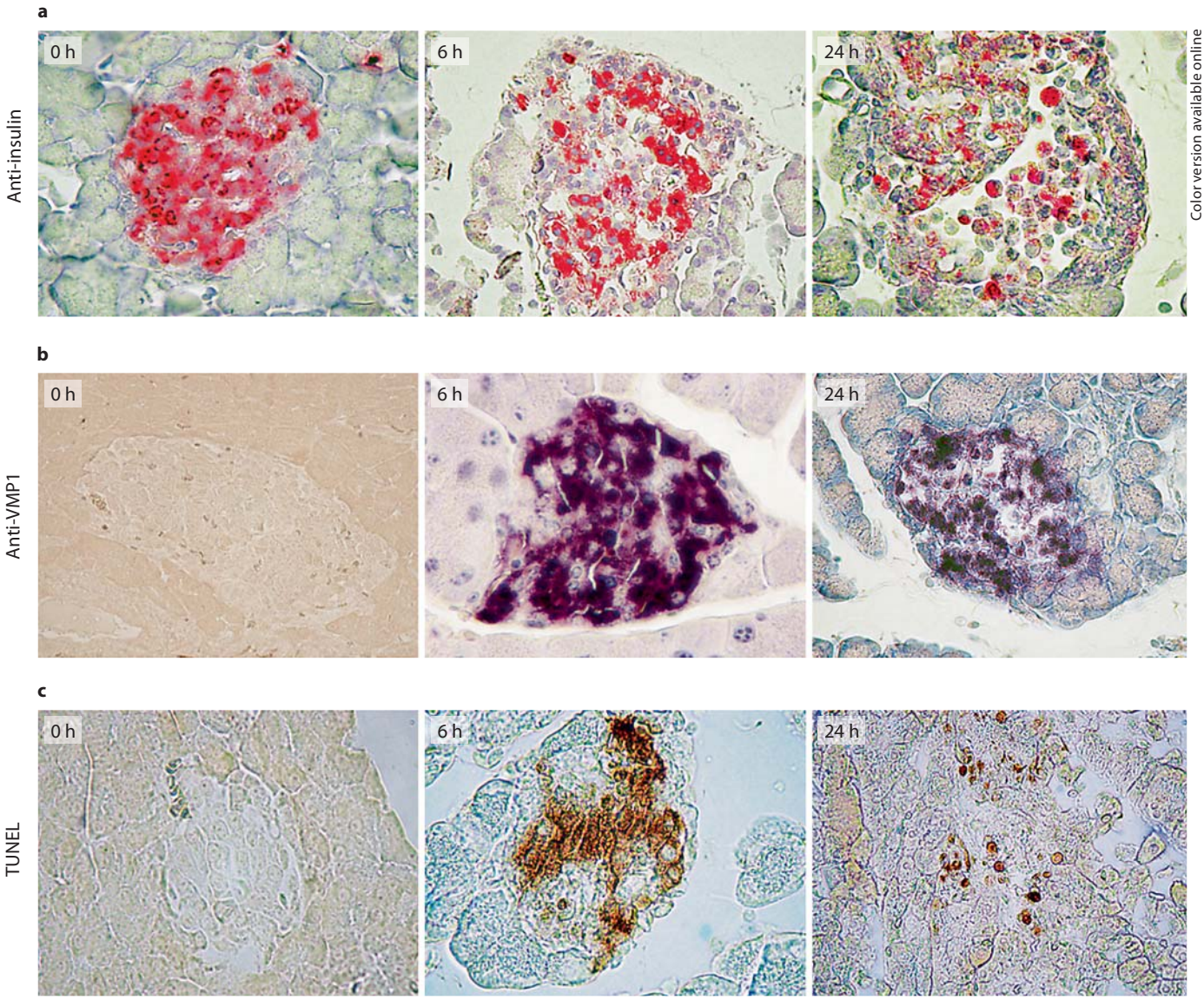

Fig. 2. VMP1 is early expressed in beta cells of STZ-treated rat pancreas. **a** Insulin immunohistochemistry. After 6 h treatment, reduced number of insulin-positive beta cells is observed compared with control tissue. After 24 h STZ treatment, a few insulin-positive cells are still present in pancreas tissue. **b** VMP1 immunohistochemistry. After 6 h treatment, maximal VMP1 immunostaining is observed in pancreas tissue from STZ-treated rats. After 24 h treatment, VMP1-positive cells are still present in the remaining beta cells. **c** TUNEL assay of STZ-treated pancreas. TUNEL-positive signal is maximal after 6 h STZ treatment and several TUNEL-positive beta cells remain after 24 h STZ treatment. Results are representative of five independent experiments.

[25–28], and this interaction is essential for autophagosome formation. We hypothesized that the early VMP1 expression in response to STZ in islet cells is related to autophagy. Therefore, we first analyzed the effects of STZ administration on morphological events in islet cells. Light-microscopic examination of thin plastic sections, stained with toluidine blue, shows chromatin aggregation and cytoplasm vacuolization in central islet cells during STZ treatment (fig. 3a). Further electron microscopy confirms the ultrastructure of autophagy in response to STZ (fig. 3b). We then performed immunofluorescence of VMP1, Beclin1 and LC3 as a specific marker of autophagy [29, 30]. Pancreas tissue section from STZ-treated rats were immunostained with specific LC3, VMP1 and Beclin1 antibodies. We found a remarkable co-localization between VMP1 and LC3 in cytoplasmic

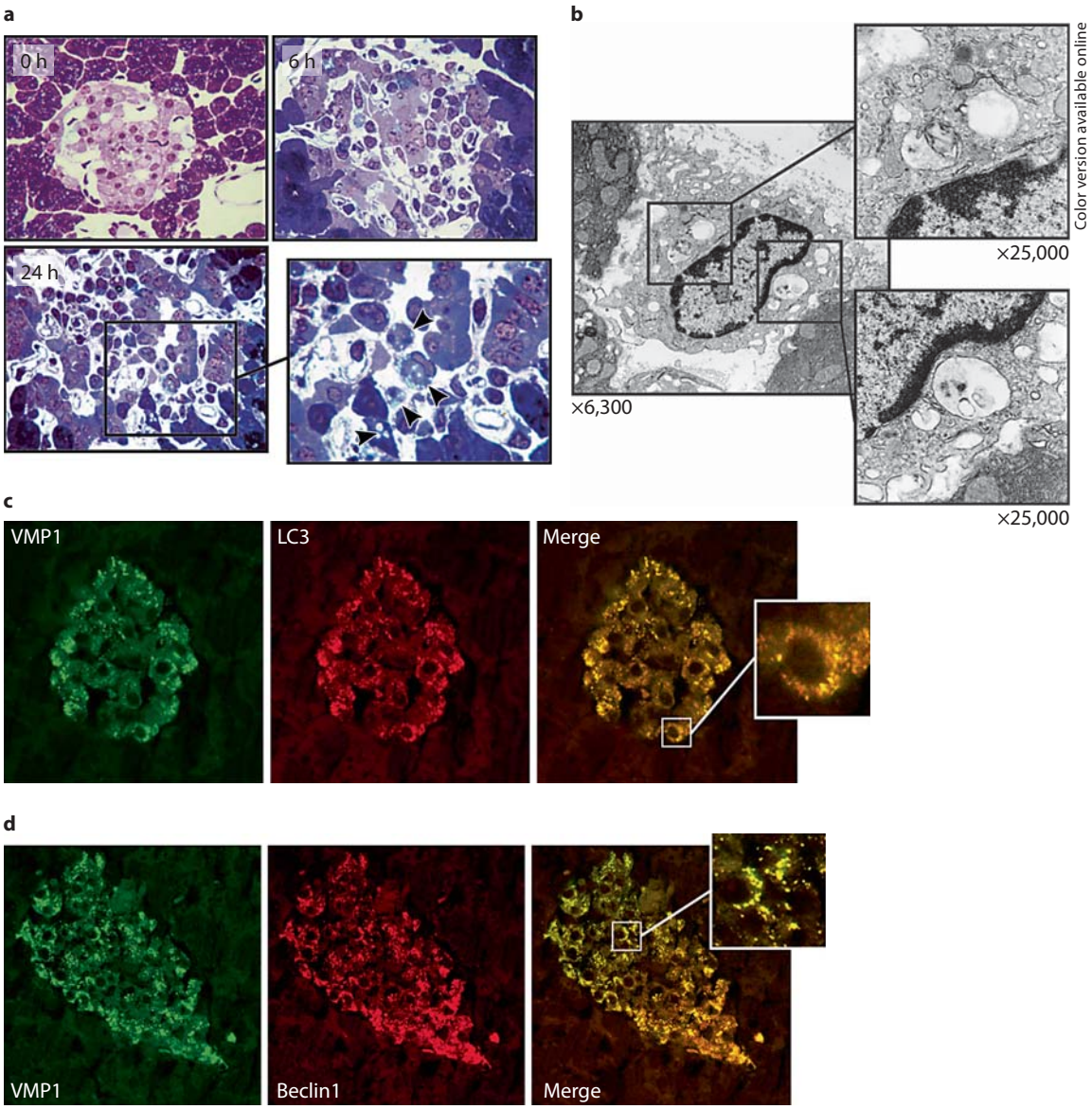

Fig. 3. VMP1 expression is related to autophagy. **a** Images of toluidine blue slices in control and after 6 and 24 h STZ-treated rat pancreas. Wide infiltration and cell loss are observed in islets from STZ-treated rat pancreas. Remaining beta cells with several cytoplasm vesicles can be visualized (arrowhead in zoom insert). **b** Electron microscopy of 24 h STZ-treated pancreas confirms autophagic ultrastructure of pancreas beta cells. Autophagic structures are detailed. **c** Immunofluorescence of VMP1 and LC3 in STZ-treated pancreas tissue. As a specific marker of autophagy, LC3-positive aggregates indicate the presence of STZ-induced VMP1-positive autophagosomes in pancreas islets. **d** VMP1 and Beclin1 immunofluorescence in STZ-treated pancreas tissue shows co-localization between both autophagy-related proteins. Results are representative of three independent experiments.

vesicles (fig. 3c), suggesting autophagy in islet cells during experimental diabetes. Moreover, we found co-localization between VMP1 and Beclin1 suggesting that both proteins may be involved in autophagosome formation (fig. 3d).

In order to know if VMP1 expression and autophagy are triggered by a direct effect of STZ on pancreatic islet cells, we treated isolated pancreas islets from untreated rats with increasing doses of STZ. Figure 4a shows the RT-PCR results. VMP1 mRNA expression is activated in response to STZ treatment in isolated pancreas islets. We also investigated VMP1 protein by immunohistochemistry and we find early and high expression of VMP1 in response to STZ treatment. VMP1 protein expression is

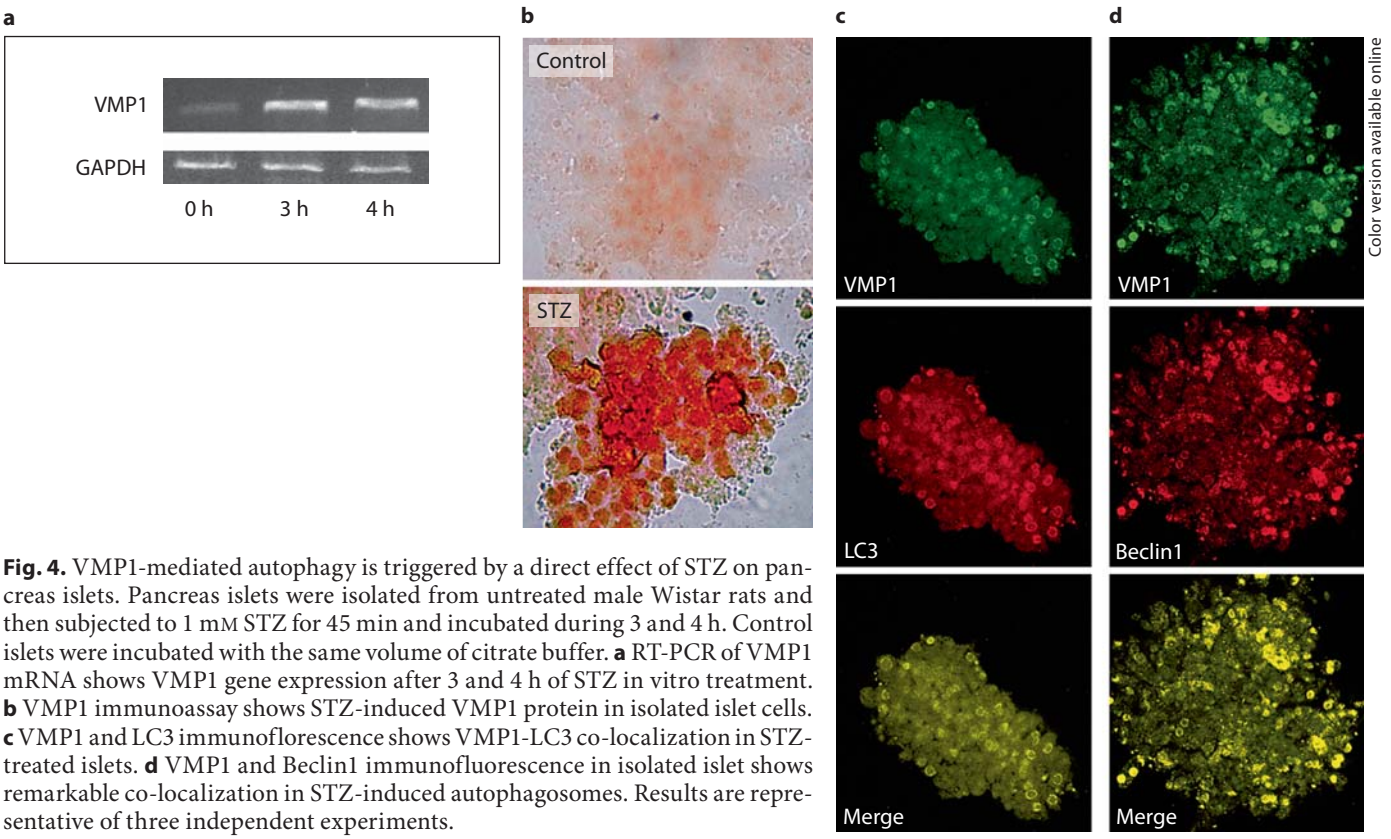

Fig. 4. VMP1-mediated autophagy is triggered by a direct effect of STZ on pancreas islets. Pancreas islets were isolated from untreated male Wistar rats and then subjected to 1 mM STZ for 45 min and incubated during 3 and 4 h. Control islets were incubated with the same volume of citrate buffer. **a** RT-PCR of VMP1 mRNA shows VMP1 gene expression after 3 and 4 h of STZ in vitro treatment. **b** VMP1 immunoassay shows STZ-induced VMP1 protein in isolated islet cells. **c** VMP1 and LC3 immunoflorescence shows VMP1-LC3 co-localization in STZ-treated islets. **d** VMP1 and Beclin1 immunofluorescence in isolated islet shows remarkable co-localization in STZ-induced autophagosomes. Results are representative of three independent experiments.

highly activated in islet cells 3 h after STZ treatment (fig. 4b) compared to untreated islets. Finally, to verify autophagy induction, we analyzed LC3 and VMP1 by immunofluorescence in STZ-treated islets. As a specific marker of autophagy, LC3-positive aggregates indicate the presence of STZ-induced autophagosomes (fig. 4c). Moreover, we find remarkable co-localization between LC3 and VMP1 and between Beclin1 and VMP1 in autophagosomal structures from STZ-treated islets (fig. 4c–d). These results confirm those found in vivo experiments and demonstrate that VMP1 expression and autophagy are activated in pancreas islet as a direct response to STZ treatment. Furthermore, our results suggest that VMP1-Beclin1 interaction is involved in the autophagic process activated in the beta cell in response to experimental diabetes.

Discussion

We show that STZ treatment induces VMP1 expression and autophagy in pancreas beta cells as early cellular events during experimental diabetes. We also demonstrate that VMP1 expression and autophagy are activated in pancreas islets as a direct response to STZ treatment. Furthermore, our results suggest that VMP1-Beclin1 interaction is involved in the autophagic process activated in beta cell in response to experimental diabetes.

VMP1 is an autophagosomal transmembrane protein which expression triggers autophagy in mammalian cells. Previous works demonstrated that VMP1 transcript is highly activated in acinar cells during experimental pancreatitis and its expression is related to acinar cell autophagy [19, 20]. Our results indicated that experimental diabetes induces early VMP1 expression and autophagy in islet cells. Notably, VMP1 expression in response to STZ is restricted to pancreas islet cells and it was not observed in exocrine pancreas. VMP1 expression is observed in cells containing insulin suggesting that beta cells undergo autophagy during experimental diabetes. Maximal expression of VMP1 is coincident with the appearance of apoptosis showing that pancreas beta cell activates VMP1 expression as an early molecular response to STZ-induced cell injury. These findings are consistent with those reported by Wada and Yagihashi [31] describing overexpression of poly(ADP ribose) polymerase (PARP) and

massive beta cell death as early as 5–8 h after single high-dose STZ injection. Therefore, early VMP1 expression and autophagy may be part of the beta cell response to experimental diabetes. STZ effects on beta cell include depletion of NAD and production of ROS [6, 7]. Interestingly, the novel drug FK866, which causes NAD depletion, was reported to induce autophagy in neuroblastoma cells [32]. Moreover, autophagic pathways appear to be regulated by ROS. Under starvation conditions, ROS are essential for autophagy to proceed because they control the activity of Atg4, a family of cysteine proteases that are necessary for autophagosome formation. Therefore, the activation of autophagy in beta cells could contribute to the deleterious effect of STZ and may be involved in the implementation of apoptotic cell death or in a way different from apoptosis.

Alternatively, autophagy can also act as a survival pathway by delaying cell death due to apoptosis, e.g. during nutrient starvation [33]. Autophagy could act as a defense mechanism against diabetes-induced cell injury. It has been suggested that autophagy may be a useful mechanism for eliminating beta-cell-damaged mitochondria induced by diabetes [34]. Moreover, diabetes-induced oxidative stress promotes ubiquitination and storage of proteins into cytoplasmic aggregates of pancreatic beta cells that may be cleared by autophagy [18]. The control of autophagy is characterized by requiring PI3P (the product of class III PI3K or hVps34) for autophagosome formation, and by being inhibited by mTOR. Antidiabetic agents such as thiazolidinediones and metformin are known activators of AMPK [35] and part of their mechanism of action may involve the stimulation of autophagy. However, the mechanism by which inhibition of mTOR or activation of AMPK leads to the autophagic process remains an enigma. Inhibition of mTOR by either amino acid depletion or rapamycin administration activates VMP1 expression and autophagy. Therefore, early VMP1 expression in response to STZ may allow autophagy to occur in order to protect beta cell against death.

In conclusion, results reported here show early VMP1 expression and autophagy induced by STZ treatment, suggesting that VMP1 expression could mediate the activation of autophagy in response to the experimental disease. Specific beta cell toxic agents such as STZ could lead to VMP1 expression in order to start the autophagic process involved in molecular mechanisms of cell damage or cell defense. Although the pathophysiological role of autophagy in pancreas diseases remains to be elucidated, VMP1 expression would reveal a common molecular pathway of cell response to diseases affecting the endocrine as well as the exocrine pancreas. Further studies leading to a better understanding of the role of VMP1 expression and autophagy in beta cells as a response to disease would be of potential clinical relevance.

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