Original Paper

Intermittent-Hypoxia-Induced Autophagy Activation Through the ER-Stress-Related PERK/eIF2 α /ATF4 Pathway is a Protective Response to Pancreatic β -Cell Apoptosis

Shuling Song^a Jin Tan^a Yuyang Miao^b Zuoming Sun^c Qiang Zhang^a^aDepartment of Geriatrics, Tianjin Medical University General Hospital, Tianjin Geriatrics Institute, Tianjin, ^bTianjin Medical University, Tianjin, China, ^cDivision of Immunology, Beckman Research Institute of the City of Hope, Duarte, USA**Key Words**Intermittent hypoxia • ER stress • Autophagy • Apoptosis • Pancreatic β -cells**Abstract**

Background/Aims: Intermittent hypoxia (IH) causes apoptosis in pancreatic β -cells, but the potential mechanisms remain unclear. Endoplasmic reticulum (ER) stress, autophagy, and apoptosis are interlocked in an extensive crosstalk. Thus, this study aimed to investigate the contributions of ER stress and autophagy to IH-induced pancreatic β -cell apoptosis. **Methods:** We established animal and cell models of IH, and then inhibited autophagy and ER stress by pharmacology and small interfering RNA (siRNA) in INS-1 cells and rats. The levels of biomarkers for autophagy, ER stress, and apoptosis were evaluated by immunoblotting and immunofluorescence. The number of autophagic vacuoles was observed by transmission electron microscopy. **Results:** IH induced autophagy activation both *in vivo* and *in vitro*, as evidenced by increased autophagic vacuole formation and LC3 turnover, and decreased SQSTM1 level. The levels of ER-stress-related proteins, including GRP78, CHOP, caspase 12, phosphorylated (p)-protein kinase RNA-like ER kinase (PERK), p-eIF2 α , and activating transcription factor 4 (ATF4) were increased under IH conditions. Inhibition of ER stress with tauroursodeoxycholic acid or 4-phenylbutyrate partially blocked IH-induced autophagy in INS-1 cells. Furthermore, inhibition of PERK with GSK2606414 or siRNA blocked the ER-stress-related PERK/eIF2 α /ATF4 signaling pathway and inhibited autophagy induced by IH, which indicates that IH-induced autophagy activation is dependent on this signaling pathway. Promoting autophagy with rapamycin alleviated IH-induced apoptosis, whereas inhibition of autophagy with chloroquine or autophagy-related gene (Atg5 and Atg7) siRNA aggravated pancreatic β -cell apoptosis caused by IH. **Conclusion:** IH induces autophagy activation through the ER-stress-related PERK/eIF2 α /ATF4 signaling pathway, which is a protective response to pancreatic β -cell apoptosis caused by IH.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Qiang Zhang

Department of Geriatrics, Tianjin Medical University General Hospital; Tianjin Geriatrics Institute, Anshan Road NO.154, Tianjin 300052 (China)
Tel. 86-22-60363659, Fax 86-22-60363659, E-Mail zhangqiangyulv@163.com

Introduction

Intermittent hypoxia (IH), which results from recurring episodes of upper airway obstruction and is a characteristic and important pathophysiological pathway of obstructive sleep apnea, is believed to be a potential major factor causing pancreatic β -cell injury that may play a pivotal role in the development and progression of glucose metabolism disorder [1, 2]. Many studies, including our previous study, have demonstrated that IH leads to pancreatic β -cell dysfunction and even to apoptosis [3-5]. However, the underlying molecular mechanisms of IH-induced pancreatic β -cell apoptosis are still unclear.

Autophagy is a cellular catabolic process that sequesters senescent or damaged proteins/subcellular organelles in autophagosomes for recycling of their products [6]. Under normal physiological conditions, autophagy contributes to the maintenance of cellular homeostasis. Under pathological conditions, autophagy is induced in response to diverse stress conditions including hypoxia, ischemia, and oxygen-glucose deprivation [7-9], which mitigates or exacerbates injury by regulating apoptosis. Furthermore, autophagy has been implicated in the pathophysiology of diabetes mellitus as it applies to β -cell dysfunction [10, 11]. Therefore, we speculated that IH, a special form of hypoxia, promotes autophagy activation that is involved in pancreatic β -cell apoptosis.

Endoplasmic reticulum (ER) stress, a common cellular stress response that is triggered by a variety of conditions that disturb cellular homeostasis, is not only associated with pancreatic β -cell dysfunction and apoptosis [12, 13], but is also closely related to autophagy activation [14, 15]. It is obvious that ER stress, autophagy, and apoptosis are interlocked in an extensive crosstalk [16]. Therefore, in the present study, we investigated the role and potential mechanisms of ER stress and autophagy in IH-induced pancreatic β -cell apoptosis.

Materials and Methods

Animals and IH exposure

Thirty 8-week-old male Sprague-Dawley rats purchased from the Chinese Academy of Military Science (Beijing, China) were used in this study. All animal experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of Tianjin Medical University. All rats were randomly allocated to six groups ($n = 5$ each): A. normoxia control group (NC); B. chloroquine treatment group (CQ), intraperitoneal injection of chloroquine (60 mg/kg/day); C. rapamycin treatment group (RA), intraperitoneal injection of rapamycin (0.5 mg/kg/day); D. IH group (IH); E. IH and chloroquine treatment group (IH+CQ), intraperitoneal injection of chloroquine (60 mg/kg/day) prior to IH exposure; and F. IH and rapamycin treatment group (IH+RA), intraperitoneal injection of rapamycin (0.5 mg/kg/day) prior to IH exposure.

The IH animal model was established according to the procedures described by He et al [17]. Briefly, model rats were exposed to IH for 6 weeks, 8 h/day (9 AM to 5 PM) in a specialized Plexiglas chamber. A gas control delivery system was designed to modulate the flow of nitrogen (N_2 , hypoxia phase, 30 s) or compressed air (air, reoxygenation phase, 90 s) alternately into the Plexiglas chamber. Gas flow and O_2 concentration in the chamber were continuously monitored by an O_2 analyzer (CY-12C, Jiande Meicheng Analysis Instrument Company, Zhejiang, China). The O_2 concentration in the chamber was rapidly decreased to 5% in the hypoxia phase, after which it was quickly increased to a maximum of 21%. After 6 weeks, all rats were anesthetized for euthanasia, and the pancreas and islets were isolated as described previously with some modifications [18].

INS-1 cell culture and IH exposure

Rat insulinoma INS-1 cells were purchased from the China Infrastructure of Cell Line Resource (Beijing, China), and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 50 μ M 2-mercaptoethanol, 10 mM HEPES, and 1 mM sodium pyruvate. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 .

INS-1 cells were exposed to IH until they reached approximately 80% confluence. The IH cell model was established according to our previously described protocol [19]. Briefly, the culture medium was changed to fresh medium before the cells were exposed to IH. INS-1 cells grown in culture plates were placed in a Plexiglas exposure chamber, which was alternately flushed with a hypoxia gas mixture (1.5% O₂, 5% CO₂, and balanced N₂, hypoxia phase, 300 s) or normoxia gas mixture (21% O₂, 5% CO₂, and balanced N₂, reoxygenation phase, 600 s). The chamber was equipped with a humidifier, thermostat, and molecular sieve to maintain an inner temperature of 37°C, humidity of 45%, and relatively germ-free conditions. O₂ concentration in the chamber was monitored by an O₂ analyzer (CY-12C, Jiande Meicheng Analysis Instrument Company).

Immunoblotting

Proteins were extracted from rat islets and INS-1 cells using a protein extraction kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. Protein concentrations were measured using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA). Protein sample and pre-stained molecular weight markers (Thermo Fisher Scientific) were applied to 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA). The membranes were blocked by 5% non-fat dry milk in Tris-buffered saline and Tween 20 for 2 h at room temperature, and then incubated with primary antibodies (Table 1) at 4°C overnight. The appropriate horseradish peroxidase-conjugated secondary IgG was incubated with the membranes for 1 h at room temperature. Chemiluminescence was imaged in a Chemi Doc XRS+ WB molecular imager using Image Lab software (Bio-Rad Laboratories, Hercules, CA), and band intensity was measured by ImageJ software.

Immunofluorescence

After the experimental conditions were established, the INS-1 cells, cultured on coverslips, were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min. Then, cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 3% bovine serum albumin in buffer for 30 min at room temperature. Subsequently, cells were incubated with anti-light chain 3B (LC3B) antibody (1:400, Cell Signaling Technology, Danvers, MA), anti-GRP78 antibody (1:500, Abcam, Cambridge, MA), and

Table 1. Antibodies for immunoblotting

Antibody	Dilution	Catalog #	Vendor	Molecular weight
LC3B	1:1000	2775	CST	14, 16 kDa
SQSTM1	1:1000	5114	CST	62 kDa
GRP78	1:1000	ab21685	abcam	75 kDa
CHOP	1:1000	2895	CST	27 kDa
Caspase12	1:1000	ab62484	abcam	42 kDa
PERK	1:1000	3192	CST	140 kDa
p-PERK (Thr980)	1:1000	3179	CST	170 kDa
eIF2α	1:1000	9722	CST	38 kDa
p-eIF2α (Ser51)	1:1000	3597	CST	38 kDa
ATF4	1:1000	11815	CST	49 kDa
Atg5	1:1000	12994	CST	55 kDa
Atg7	1:1000	8558	CST	78 kDa
Cleaved caspase3	1:1000	9664	CST	17 kDa
GAPDH	1:1000	2118	CST	37 kDa
Peroxidase-Conjugated anti-Rabbit IgG (H + L)	1:5000	ZB-2305	ZSGB-BIO	
Peroxidase-Conjugated anti-Mouse IgG (H + L)	1:5000	ZB-2301	ZSGB-BIO	

anti-beta III tubulin antibody (1:200, Abcam) at 4°C overnight. The appropriate FITC-labeled secondary antibody was incubated with the slides for 1 h at room temperature. Apoptosis was assessed with a TdT-dUTP nick-end labeling (TUNEL) apoptosis assay kit (Roche, Indianapolis, IN). The slides were incubated with TUNEL reaction mixture for 1 h at 37°C. Finally, slides were washed and nuclei were stained with DAPI. Images were obtained using a confocal microscope (FV 1200, Olympus, Tokyo, Japan) and cellSens software (Olympus). LC3-II-positive dots were counted in individual INS-1 cells, and the average of dots in at least 30 cells is presented in the figures.

Transmission electron microscopy

Rat pancreas and INS-1 cells were cut into smaller pieces and fixed in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and 2% formaldehyde (2 h), post-fixed in 1% osmium tetroxide (2 h), dehydrated in an ascending series of ethanol, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed using an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Small interfering RNA transfection

RNA interference was performed by transient transfection of cells with small interfering RNA (siRNA) against the designated genes using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Rat protein kinase RNA-like ER kinase (PERK) siRNA, Atg5 siRNA, Atg7 siRNA, and negative control siRNA were purchased from GenePharma (Shanghai, China).

Statistical analysis

All statistical tests were performed with SPSS 16.0 (SPSS Inc., Chicago, IL). All data are based on at least three independent experiments and are expressed as the mean $\pm$ standard error of the mean (SEM). Results between groups were compared by Student's *t* test or one-way analysis of variance. A *p*-value less than 0.05 was considered significant.

Results

IH induces autophagy activation and ER stress in rat pancreas

To investigate whether autophagy is associated with apoptosis caused by IH, we established an animal model of IH and examined the extent of autophagy in pancreatic islets. Immunoblotting results showed that the level of LC3-II was increased significantly in the IH group when compared with the normoxia control group (Fig. 1a, b). We also observed the autophagic vacuoles by transmission electron microscopy, and found that the number of autophagic vacuoles was increased after IH treatment (Fig. 1f, g). The above results suggest that IH increases the level of autophagy in rat pancreas.

The dynamic process of autophagy includes initiation, elongation, maturation, and degradation, also called autophagic flux. LC3-I to LC3-II turnover and SQSTM1 protein levels are regarded as the principal markers to monitor autophagic flux [20]. Our results showed that the level of SQSTM1 was decreased (Fig. 1a, c), and the LC3 turnover was increased in rat pancreas exposed to IH (Fig. 1d, e). To further examine the autophagic flux *in vivo*, the rats were treated with the autophagy inhibitor chloroquine and the autophagy activator rapamycin before IH exposure. Compared with IH treatment, chloroquine and rapamycin pretreatment further upregulated LC3-II/LC3-I levels (Fig. 1d, e). All of these results suggest that IH induces autophagy activation rather than blocking the fusion of autophagosomes with lysosomes.

To investigate the potential mechanisms of autophagy activation under IH, we initially assessed the expression of ER-stress-related proteins in rat pancreas. The levels of GRP78, CHOP, and caspase 12 (Fig. 1h) were increased significantly in the IH group compared with the normoxia control group.

Fig. 1. IH induces autophagy activation and ER stress in rat pancreas. The expression of LC3-II and SQSTM1 was analyzed by immunoblotting (a) in the NC and IH groups, and band quantification was performed using ImageJ software (b, c). Rats were pretreated with CQ and RA before hypoxia exposure each day. The LC3-II/LC3-I ratio was detected by immunoblotting (d), and band quantification was performed using ImageJ software (e). Autophagic vacuoles in rat pancreas were detected by transmission electron microscopy (f). Scale bars: 1 μ m (NC) and 2 μ m (IH). Quantification of the number of autophagic vacuoles from at least 20 randomly selected areas is shown (g). The marker proteins of ER stress were assayed by immunoblotting (h) in the NC and IH groups, and i (GRP78), j (CHOP), and k (caspase 12) show the densitometry of each band. All rats were randomly grouped (n = 5 each). Data are depicted as the mean $\pm$ SEM from three independent experiments. CQ, chloroquine, 60 mg/kg; RA, rapamycin, 0.5 mg/kg. *p<0.05, **p<0.01.

IH induces autophagy activation and ER stress in INS-1 cells

IH induces autophagy activation *in vivo*. To further investigate the potential mechanisms of autophagy activation induced by IH, we established a cell model of IH *in vitro*. The results of immunoblotting and immunofluorescence showed that the level of LC3-II increased with the duration of IH, especially in the 12-h IH group (Fig. 2a, b, c, d). We also observed the autophagic vacuoles by transmission electron microscopy, and found that the number of

Fig. 2. IH induces autophagy activation in INS-1 cells. (a) The expression of LC3-II was analyzed by immunoblotting in NC, 4-h IH (IH4h), IH8h, and IH12h groups, and band quantification was performed using ImageJ software (b). (c) Expression of LC3-II was analyzed by immunofluorescence in the NC and IH12h group, and quantification of LC3-II-positive dots is depicted in d. Scale bar: 10 μ m. Autophagic vacuoles in INS-1 cells were detected by transmission electron microscopy (e). Scale bars in the NC group represent 2 μ m and 1 μ m. Scale bars in the IH group represent 5 μ m, 1 μ m, and 2 μ m. Quantification of the number of autophagic vacuoles from at least 20 randomly selected areas is shown (f). Expression of SQSTM1 was analyzed by immunoblotting in the NC, IH4h, IH8h, and IH12h groups (g), and band quantification was performed using ImageJ software (h). INS-1 cells were pretreated with CQ and RA before IH exposure for 12 h. The LC3-II/LC3-I ratio was detected by immunoblotting (i), and band quantification was performed using ImageJ software (j). Data are depicted as the mean $\pm$ SEM from three independent experiments. CQ, chloroquine, 10 μ M; RA, rapamycin, 50 nM. *p<0.05, **p<0.01.

autophagic vacuoles was increased after IH treatment (Fig. 2e, f). The above results suggest that IH increases the level of autophagy in INS-1 cells.

We also found that the level of SQSTM1 decreased with the duration of IH, especially in the 12-h IH group (Fig. 2g, h), and the LC3 turnover was increased in INS-1 cells exposed to IH (Fig. 2i, j). To further examine the autophagic flux *in vitro*, the INS-1 cells were treated with the autophagy inhibitor chloroquine and the autophagy activator rapamycin before IH. Compared with IH treatment, chloroquine and rapamycin pretreatment further upregulated LC3-II/LC3-I levels (Fig. 2i, j). All of these results suggest that IH induces autophagy activation in INS-1 cells.

GRP78, CHOP, and caspase 12 are biomarkers of ER stress. The immunofluorescence results showed that IH upregulated the level of GRP78 in INS-1 cells (Fig. 3c). Immunoblotting showed that the levels of GRP78 (Fig. 3a, b), CHOP (Fig. 3d, e), and caspase 12 (Fig. 3f, g) increased with the duration of IH, especially in the 12-h IH group, indicating that IH induces ER stress.

Fig. 3. IH induces ER stress in INS-1 cells. The marker proteins of ER stress were assayed by immunoblotting (a, d, f) in the NC, IH4h, IH8h, and IH12h groups, and b (GRP78), e (CHOP), and g (caspase 12) show the densitometry of each band. (c) Expression of GRP78 was analyzed by immunofluorescence in the NC and IH12h groups. Scale bar: 20 μ m. Data are depicted as the mean $\pm$ SEM from three independent experiments. *p<0.05, **p<0.01.

IH-induced autophagy is ER stress dependent

Our initial results showed that IH induces autophagy activation and ER stress. To examine whether the autophagy was activated after ER stress induction, INS-1 cells were exposed to the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) or 4-phenylbutyrate (4-PBA) before IH exposure. As shown in the Fig. 4a, TUDCA strongly reduced the expression levels of GRP78, CHOP, and caspase 12 in IH-treated cultures. The IH-induced increase of

Fig. 4. IH-induced autophagy is ER stress dependent. (a) INS-1 cells were treated with ER stress inhibitor TUDCA and cultured with IH for 12 h. The expression of ER stress marker proteins and autophagy marker proteins was detected by immunoblotting, and the band quantification of GRP78 (b), CHOP (c), caspase 12 (d), LC3-II (e), and SQSTM1 (f) was performed using ImageJ software. (g) INS-1 cells were treated with ER stress inhibitor 4-PBA and cultured with IH for 12 h. The expression of ER stress marker proteins and autophagy marker proteins was detected by immunoblotting, and the band quantification of GRP78 (h), CHOP (i), caspase 12 (j), LC3-II (k), and SQSTM1 (l) was performed using ImageJ software. Data are depicted as the mean $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

LC3-II and decrease of SQSTM1 levels were alleviated by TUDCA (Fig. 4a, e, f). Similar to the results of TUDCA intervention, the ER stress was mitigated by 4-PBA (Fig. 4g, h, i, j), which was accompanied with autophagy reduction (Fig. 4g, k, l). Taken together, these findings demonstrate that IH-induced autophagy is ER stress dependent.

IH activates autophagy through the ER-stress-related PERK/eIF2 α /activating transcription factor 4 signaling pathway

Activating transcription factor 4 (ATF4) is a transcription factor induced under severe hypoxia and a component of the PERK pathway involved in autophagy induced by ER stress [21, 22]. Therefore, we assessed changes in the levels of these proteins, to explore whether the PERK/eIF2 α /ATF4 signaling pathway is involved in autophagy activation under IH conditions. As shown in Fig. 5a, IH increased the expression levels of phosphorylated (p)-PERK, p-eIF2 α , and ATF4 in a time-dependent manner. We further used a PERK inhibitor before IH treatment, and found that treatment with the PERK inhibitor significantly blocked LC3-II biosynthesis as well as the increased expression of p-PERK, p-eIF2 α , and ATF4 induced by IH (Fig. 5e). Moreover, transfection with PERK siRNA successfully reduced the level of p-PERK, p-eIF2 α , and ATF4 (Fig. 6c), as well as IH-induced LC3-II (Fig. 6g). These data indicate that IH activates autophagy through the ER-stress-related PERK/eIF2 α /ATF4 signaling pathway.

Fig. 5. Blocking the PERK/eIF2 α /ATF4 signaling pathway with GSK2606414 mitigates autophagy induced by IH. INS-1 cells were treated under NC, IH4h, IH8h, and IH12h conditions. The expression of PERK/p-PERK, eIF2 α /p-eIF2 α , and ATF4 in INS-1 was assayed by immunoblotting (a), and the band quantification of p-PERK (b), p-eIF2 α (c), and ATF4 (d) was performed using ImageJ software. INS-1 cells were treated with GSK2606414, and then cultured with IH for 12 h. The expression of PERK/p-PERK, eIF2 α /p-eIF2 α , ATF4 (e), and LC3-II (i) in INS-1 was assayed by immunoblotting, and the band quantification of p-PERK (f), p-eIF2 α (g), ATF4 (h), and LC3-II (j) was performed using ImageJ software. Data are depicted as the mean $\pm$ SEM from three independent experiments. * p <0.05, ** p <0.01.

Fig. 6. Blocking the PERK/eIF2 α /ATF4 signaling pathway with PERK siRNA mitigates autophagy induced by IH. INS-1 cells were treated with vehicle, control siRNA, or PERK siRNA. PERK expression was detected by immunoblotting (a), and band quantification was performed using ImageJ software (b). INS-1 cells were treated with PERK siRNA, and then cultured with IH for 12 h. The expression of PERK/p-PERK, eIF2 α /p-eIF2 α , ATF4 (c), and LC3-II (g) in INS-1 was assayed by immunoblotting, and the band quantification of p-PERK (d), p-eIF2 α (e), ATF4 (f), and LC3-II (h) was performed using ImageJ software. Data are depicted as the mean $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Autophagy activation ameliorates pancreatic β -cell apoptosis caused by IH

The apoptotic protein cleaved caspase 3 and the percentage of apoptotic cells were increased with the duration of IH, which indicated that IH caused INS-1 cell apoptosis (Fig. 7a, c). To clarify the role of autophagy in IH-induced cell apoptosis, we manipulated autophagy using chloroquine and rapamycin, which work by blocking or promoting autophagic flux, respectively. Chloroquine and rapamycin pretreatment further increased the level of LC3-II induced by IH (Fig. 7e, f), demonstrating the efficiency of the two drugs in IH-induced autophagy. The level of cleaved caspase 3 was further increased by chloroquine treatment, while it was decreased by rapamycin treatment (Fig. 7e, g). We further blocked autophagy by siRNA against the autophagy-related genes Atg5 and Atg7, and the efficiency is shown in Fig. 8a and Fig. 8c. Similar to the results of chloroquine treatment, knockdown of Atg5 and Atg7 also suppressed the expression of LC3-II, and promoted the INS-1 cell apoptosis caused by IH (Fig. 8e, i). These results suggest that autophagy activation ameliorates IH-induced apoptosis *in vitro*.

Fig. 7. Autophagy activation ameliorates INS-1 cell apoptosis caused by IH. INS-1 cells were treated under NC, 4-h IH (IH4h), IH8h, and IH12h conditions. The expression of cleaved caspase 3 was assayed by immunoblotting (a), and band quantification was performed using ImageJ software (b). Cell apoptosis was determined by TUNEL assay (c), and quantification of apoptotic cells is depicted in d. Scale bar: 50 μ m. INS-1 cells were pretreated with CQ and RA, and then cultured with IH for 12 h. The expression of LC3-II and cleaved caspase 3 was analyzed by immunoblotting (e), and band quantification of LC3-II (f) and cleaved caspase 3 (g) was performed using ImageJ software. Data are depicted as the mean $\pm$ SEM from three independent experiments. *p<0.05, **p<0.01.

IH also promotes pancreatic β -cell apoptosis *in vivo*, which was demonstrated by the increased level of cleaved caspase 3 in the IH group (Fig. 9a). Furthermore, inhibition of autophagy by chloroquine increased the level of cleaved caspase 3, and promoting autophagy with rapamycin decreased the level of cleaved caspase 3 (Fig. 9c). These results indicate that autophagy activation alleviates IH-induced apoptosis *in vivo*. Taking these results into consideration, we conclude that autophagy activation ameliorates pancreatic β -cell apoptosis caused by IH.

Fig. 8. siRNA targeting autophagy-related genes aggravates INS-1 cell apoptosis caused by IH. INS-1 cells were treated with vehicle, control siRNA, Atg5 siRNA, or Atg7 siRNA. The expression of Atg5 (a) and Atg7 (c) was detected by immunoblotting, and band quantification was performed using ImageJ software (b, d). INS-1 cells were treated with Atg5 siRNA, and then cultured with IH for 12 h. The expression of Atg5, LC3-II, and cleaved caspase 3 was analyzed by immunoblotting (e), and the band quantification of Atg5 (f), LC3-II (g), and cleaved caspase 3 (h) was performed using ImageJ software. INS-1 cells were treated with Atg7 siRNA, and then cultured with IH for 12 h. The expression of Atg7, LC3-II, and cleaved caspase 3 was analyzed by immunoblotting (i), and the band quantification of Atg5 (j), LC3-II (k), and cleaved caspase 3 (l) was performed using ImageJ software. Data are depicted as the mean $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Discussion

The pivotal role of IH-induced pancreatic β -cell injury in the development and progression of glucose metabolism disorder has been shown [3-5], but the potential mechanisms of pancreatic β -cell injury are largely unknown. In the present study, we found that IH induces autophagy activation through the ER-stress-related PERK/eIF2 α /ATF4 signaling pathway, which is a protective response to pancreatic β -cell apoptosis caused by IH. However, autophagy is not sufficient to resist IH-induced pancreatic β -cell apoptosis.

We established *in vivo* and *in vitro* IH models, and found that the autophagic marker protein LC3-II and autophagic vacuoles in rat islets and INS-1 cells were increased after IH treatment, which suggests that autophagy may be activated by IH in pancreatic β -cells. However, autophagy is a dynamic process that includes initiation, formation, maturation, and degradation of autophagosomes—a dynamic flow defined as autophagic flux [23]. Increased expression of LC3-II or visualization of autophagic vacuoles may result from either an enhancement of autophagosomal formation or inhibition of autophagosomal degradation [24]. Therefore, we examined autophagic flux after exposure to intermittent hypoxia, and found that the expression level of SQSTM1, a well-known autophagic substrate [25], was

Fig. 9. Autophagy activation ameliorates IH-induced apoptosis in rat pancreas. The expression of cleaved caspase 3 was analyzed by immunoblotting in the NC and IH groups (a), and band quantification was performed using ImageJ software (b). Rats were pretreated with CQ and RA before hypoxia exposure each day. The expression of LC3-II and cleaved caspase 3 was analyzed by immunoblotting (c), and band quantification of LC3-II (d) and cleaved caspase3 (e) was done by ImageJ software. (f) Schematic diagram illustrating the potential mechanism and the role of autophagy activation in IH-induced pancreatic β-cell apoptosis. All rats were randomly grouped (n = 5 each). Data are depicted as the mean ± SEM from three independent experiments. *p<0.05, **p<0.01.

decreased in pancreatic β-cells after IH treatment. Furthermore, the LC3-II/LC3-I ratio was significantly increased after IH exposure, and the ratio was higher in the chloroquine or rapamycin pretreatment group than in the IH group. The above data suggest that IH increases the formation/maturation of autophagosomes rather than blocking the fusion of the autophagosomes and lysosomes.

Consistent with our previous results, IH increased the level of cleaved caspase 3 and the percentage of apoptotic cells. To clarify whether autophagy activation is involved in pancreatic β-cell apoptosis caused by IH, we inhibited autophagy and detected pancreatic β-cell apoptosis. Our experimental results *in vivo* and *in vitro* showed that inhibition of autophagy by chloroquine significantly increased the expression of cleaved caspase 3, while promoting autophagy with rapamycin decreased the level of cleaved caspase 3 caused by IH. In addition, knockdown of autophagy-related genes also increased the IH-induced apoptosis. These data suggest that autophagy activation ameliorates the pancreatic β-cell apoptosis caused by IH.

Previous studies also demonstrated the protective role of autophagy in pancreatic β -cells under different stressors such as diabetes, palmitate, and cholesterol [26-28]. Cholesterol enhanced autophagic activity, and inhibition of autophagy aggravated cholesterol-induced pancreatic β -cell apoptosis [28]. The positive effects of autophagy in stress conditions may be associated with its ability to remove damaged proteins and subcellular organelles. However, other studies also found negative effects of autophagy on pancreatic β -cells. Tanemura et al [29]. found that rapamycin induced upregulation of autophagy, accompanied with the downregulation of insulin production and an increased percentage of apoptotic cells, which mediated islet dysfunction. They also demonstrated that 3-methyladenine, an autophagy inhibitor, ameliorated rapamycin-related β -cell dysfunction both *in vitro* and *in vivo*. The pro-apoptotic role of autophagy may be associated with the hyperstimulation-induced excessive activation of autophagy, which results in detrimental self-cannibalism beyond the possibility of cellular recovery. IH-induced pancreatic β -cell dysfunction, including augmented basal insulin secretion, insulin resistance, defective proinsulin processing, and impaired glucose-stimulated insulin secretion, has been demonstrated by several studies. Moreover, apoptosis is a more serious injury than cell dysfunction. Thus, in the present study, we examined the effect of autophagy on pancreatic β -cell apoptosis in IH conditions, though we did not examine the effect of autophagy on pancreatic β -cell dysfunction.

In pancreatic β -cells, the ER is the crucial site for insulin biosynthesis, as this is where the protein-folding machinery for secretory proteins is localized [30]. Perturbation of ER function in pancreatic β -cells, such as that caused by hypoxia, virus infection, and high levels of glucose, can lead to an imbalance in protein homeostasis and ER stress, with glucose metabolism disorder as a consequence [31, 32]. In this study, we monitored the effect of IH on ER stress in pancreatic β -cells, and found that the expression of ER stress marker proteins including GRP78, CHOP, and caspase 12 was increased in pancreatic β -cells exposed to IH. To investigate the role of ER stress in IH-induced autophagy, we pretreated pancreatic β -cells with TUDCA and 4-PBA, and found that IH-induced autophagy was decreased significantly, which indicated that IH-induced autophagy is ER stress dependent in pancreatic β -cells. Previous studies in pancreatic β -cells have demonstrated that ER stress can activate autophagy [33, 34]. In those studies, TUDCA or 4-PBA was commonly used for inhibiting ER stress [35, 36]. Recently, some studies have discovered that ER stress also induces ER-phagy, a new branch of macroautophagy that involves the generation of autophagosomes that selectively include ER membranes and whose delimiting double membranes also derive, at least in part, from the ER [37, 38]. However, in this study, the type of autophagy induced by ER stress is still unclear and needs further study.

Upon ER stress, cells activate a series of complementary adaptive mechanisms to cope with protein-folding alterations, which are known as the unfolded protein response (UPR) and ER-associated degradation [39, 40]. The UPR is characterized by three major canonical branches: inositol-requiring protein-1 α , PERK, and ATF6, which have been demonstrated to regulate autophagy at different stages in the process [37, 41, 42].

ATF4 is the main transcriptional regulator of cellular hypoxia, and the PERK/eIF2 α /ATF4 arm is the main autophagic signaling pathway of the cellular hypoxic response to ER stress [21]. Under hypoxia, the activation of PERK leads to phosphorylation of eIF2 α and activation of downstream transducers, including ATF4, that was shown to induce autophagy activation by transcriptionally regulating ATG genes [43, 44]. Rouschop et al [45]. found that hypoxia increased the expression of LC3B through the transcription factor ATF4, which is regulated by PERK, while cells deficient in PERK signaling failed to transcriptionally induce LC3B. These results suggested that the PERK/eIF2 α /ATF4 arm of the UPR is required for autophagy in hypoxic regions of tumors. Simultaneously, we found that IH, a special form of hypoxia, also increased the expression of p-PERK, p-eIF2 α , and ATF4 in pancreatic β -cells. To explore whether the PERK/eIF2 α /ATF4 signaling pathway mediates IH-induced autophagy, we further inhibited PERK by GSK2606414 or siRNA, and found that the expression of p-PERK, p-eIF2 α , and ATF4 was decreased, with a consequent reduction in autophagic activity in INS-1 cells exposed to IH. Our results also suggest that IH activates autophagy through the ER-stress-related PERK/eIF2 α /ATF4 signaling pathway in INS-1 cells.

Conclusion

In summary, we show that IH induces autophagy activation through the ER-stress-related PERK/eIF2 α /ATF4 signaling pathway in pancreatic β -cells. We also show that the activation of autophagy is a protective response to IH-induced pancreatic β -cell apoptosis (Fig. 9f). This study is an initial step in exploring the role of autophagy in pancreatic β -cell apoptosis caused by IH and may aid in future research that aims to mitigate IH-induced apoptosis in pancreatic β -cells.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81670086 and 81370183), Tianjin Natural Science Foundation (Grant No. 14JCYBJC27800), and a major special project for the prevention and control of chronic diseases in Tianjin (Grant No. 17ZXMFSY00080).

Disclosure Statement

The authors declare that they have no conflict of interest.

References

- 1 Briançon-Marjollet A, Weiszenstein M, Henri M, Thomas A, Godin-Ribuot D, Polak J: The impact of sleep disorders on glucose metabolism: endocrine and molecular mechanisms. *Diabetol Metab Syndr* 2015;7:25.
- 2 Kent BD, McNicholas WT, Ryan S. Insulin resistance, glucose intolerance and diabetes mellitus in obstructive sleep apnoea: *J Thorac Dis* 2015;7:1343-1357.
- 3 Polak J, Shimoda LA, Drager LF, Udem C, McHugh H, Polotsky VY, Punjabi NM: Intermittent hypoxia impairs glucose homeostasis in C57BL6/J mice: partial improvement with cessation of the exposure. *Sleep* 2013;36:1483-1490;1490A-1490B.
- 4 Sherwani SI, Aldana C, Usmani S, Adin C, Kotha S, Khan M, Eubank T, Scherer PE, Parinandi N, Magalang UJ: Intermittent hypoxia exacerbates pancreatic β -cell dysfunction in a mouse model of diabetes mellitus. *Sleep* 2013;36:1849-1858.
- 5 Fang Y, Zhang Q, Tan J, Li L, An X, Lei P: Intermittent hypoxia-induced rat pancreatic β -cell apoptosis and protective effects of antioxidant intervention. *Nutr Diabetes* 2014;4:e131.
- 6 Mizushima N: Autophagy: process and function. *Genes Dev* 2007;21:2861-2873.
- 7 Yang X, Yin H, Zhang Y, Li X, Tong H, Zeng Y, Wang Q, He W: Hypoxia-induced autophagy promotes gemcitabine resistance in human bladder cancer cells through hypoxia-inducible factor 1 α activation. *Int J Oncol*. 2018;53:215-224.
- 8 Peng C, Rao W, Zhang L, Gao F, Hui H, Wang K, Dai S, Yang Y, Luo P, Ma Y, Ma W, Yu X, Fei Z: Mitofusin 2 Exerts a Protective Role in Ischemia Reperfusion Injury Through Increasing Autophagy. *Cell Physiol Biochem* 2018;46:2311-2324.
- 9 Zhang Y, Fang M, Sun Y, Zhang T, Shi N, Li J, Jin L, Liu K, Fu J: Curcumin attenuates cerebral ischemia injury in Sprague-Dawley rats and PC12 cells by suppressing overactivated autophagy. *J Photochem Photobiol B* 2018;184:16.
- 10 Quan W, Hur KY, Lim Y, Oh SH, Lee JC, Kim KH, Kim GH, Kim SW, Kim HL, Lee MK, Kim KW, Kim J, Komatsu M, Lee MS: Autophagy deficiency in beta cells leads to compromised unfolded protein response and progression from obesity to diabetes in mice. *Diabetologia* 2012;55:392-403.
- 11 Gonzalez CD, Lee MS, Marchetti P, Pietropaolo M, Towns R, Vaccaro MI, Watada H, Wiley JW: The emerging role of autophagy in the pathophysiology of diabetes mellitus. *Autophagy* 2011;7:2-11.
- 12 Fonseca SG, Burcin M, Gromada J, Urano F: Endoplasmic reticulum stress in beta-cells and development of diabetes. *Curr Opin Pharmacol* 2009;9:763-770.

- 13 Guerrero-Hernández A, Leon-Aparicio D, Chavez-Reyes J, Olivares-Reyes JA, DeJesus S: Endoplasmic reticulum stress in insulin resistance and diabetes. *Cell Calcium* 2014;56:311-322.
- 14 Rashid HO, Yadav RK, Kim HR, Chae HJ: ER stress: Autophagy induction, inhibition and selection. *Autophagy* 2015;11:1956-1977.
- 15 Bartolome A, Guillen C, Benito M: Autophagy plays a protective role in endoplasmic reticulum stress-mediated pancreatic β cell death. *Autophagy* 2012;8:1757-1768.
- 16 Song S, Tan J, Miao Y, Li M, Zhang Q: Crosstalk of autophagy and apoptosis: Involvement of the dual role of autophagy under ER stress. *J Cell Physiol* 2017;232:2977-2984.
- 17 He Q, Yang QC, Zhou Q, Zhu H, Niu WY, Feng J, Wang Y, Cao J, Chen BY: Effects of varying degrees of intermittent hypoxia on proinflammatory cytokines and adipokines in rats and 3T3-L1 adipocytes. *PLoS One* 2014;9:e86326.
- 18 Li DS, Yuan YH, Tu HJ, Liang QL, Dai LJ: A protocol for islet isolation from mouse pancreas. *Nat Protoc* 2009;4:1649-1652.
- 19 Song S, Tan J, Miao Y, Zhang Q: Effect of different levels of intermittent hypoxia on autophagy of hippocampal neurons. *Sleep Breath* 2017;2:791-798.
- 20 Mizushima N, Yoshimori T, Levine B: Methods in mammalian autophagy research. *Cell* 2010;140:313-326.
- 21 Rzymiski T, Milani M, Pike L, Buffa F, Mellor HR, Winchester L, Pires I, Hammond E, Ragoussis I, Harris AL: Regulation of autophagy by ATF4 in response to severe hypoxia. *Oncogene* 2010;29:4424-4435.
- 22 B'chir W, Maurin AC, Carraro V, Averous J, Jousse C, Muranishi Y, Parry L, Stepien G, Fournoux P, Bruhat A: The eIF2 α /ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res* 2013;41:7683-7699.
- 23 Esteban-Martínez L, Boya P: Autophagic flux determination *in vivo* and *ex vivo*. *Methods* 2015;75:79-86.
- 24 Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A et al: Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 2016;12:1-222.
- 25 Katsuragi Y, Ichimura Y, Komatsu M: p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J* 2015;282:4672-4678.
- 26 Bachar-Wikstrom E, Wikstrom JD, Ariav Y, Tirosh B, Kaiser N, Cerasi E, Leibowitz G: Stimulation of autophagy improves endoplasmic reticulum stress-induced diabetes. *Diabetes* 2013;62:1227-1237.
- 27 Choi SE, Lee SM, Lee YJ, Li LJ, Lee SJ, Lee JH, Kim Y, Jun HS, Lee KW, Kang Y: Protective role of autophagy in palmitate-induced INS-1 β -cell death. *Endocrinology* 2009;150:126-134.
- 28 Wu J, Kong F, Pan Q, Du Y, Ye J, Zheng F, Li H, Zhou J: Autophagy protects against cholesterol-induced apoptosis in pancreatic β -cells. *Biochem Biophys Res Commun* 2017;482:678-685.
- 29 Tanemura M, Ohmura Y, Deguchi T, Machida T, Tsukamoto R, Wada H, Kobayashi S, Marubashi S, Eguchi H, Ito T, Nagano H, Mori M, Doki Y: Rapamycin causes upregulation of autophagy and impairs islets function both *in vitro* and *in vivo*. *Am J Transplant* 2012;12:102-114.
- 30 Berridge MJ: The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 2002;32:235-249.
- 31 Eizirik DL, Cardozo AK, Cnop M: The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* 2008;29:42-61.
- 32 Walter P, Ron D: The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011;334:1081-1086.
- 33 Kong FJ, Wu JH, Sun SY, Zhou JQ: The endoplasmic reticulum stress/autophagy pathway is involved in cholesterol-induced pancreatic β -cell injury. *Sci Rep* 2017;7:44746.
- 34 Wu W, Yao X, Jiang L, Zhang Q, Bai J, Qiu T, Yang L, Gao N, Yang G, Liu X, Chen M, Sun X: Pancreatic islet-autonomous effect of arsenic on insulin secretion through endoplasmic reticulum stress-autophagy pathway. *Food Chem Toxicol* 2018;111:19-26.
- 35 Zhu Q, Zhong JJ, Jin JF, Yin XM, Miao H: Tauroursodeoxycholate, a chemical chaperone, prevents palmitate-induced apoptosis in pancreatic β -cells by reducing ER stress. *Exp Clin Endocrinol Diabetes* 2013;121:43-47.
- 36 Wang X, Lin P, Li Y, Xiang C, Yin Y, Chen Z, Du Y, Zhou D, Jin Y, Wang A: Brucella suis Vaccine Strain 2 Induces Endoplasmic Reticulum Stress that Affects Intracellular Replication in Goat Trophoblast Cells *In vitro*. *Front Cell Infect Microbiol* 2016;6:19.
- 37 Song S, Tan J, Miao Y, Zhang Q: Crosstalk of ER stress-mediated autophagy and ER-phagy: Involvement of UPR and the core autophagy machinery. *J Cell Physiol* 2018;233:3867-3874.

- 38 Khaminets A, Heinrich T, Mari M, Grumati P, Huebner AK, Akutsu M, Liebmann L, Stolz A, Nietzsche S, Koch N, Mauthe M, Katona I, Qualmann B, Weis J, Reggiori F, Kurth I, Hübner CA, Dikic I: Regulation of endoplasmic reticulum turnover by selective autophagy. *Nature* 2015;522:354-358.
- 39 Schröder M, Kaufman RJ: ER stress and the unfolded protein response. *Mutat Res* 2005;569:29-63.
- 40 Fujita E, Kouroku Y, Isoai A, Kumagai H, Misutani A, Matsuda C, Hayashi YK, Momoi T: Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). *Hum Mol Genet* 2007;16:618-629.
- 41 Senft D, Ronai ZA: UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem Sci* 2015;40:141-148.
- 42 Rashid HO, Yadav RK, Kim HR, Chae HJ: ER stress: Autophagy induction, inhibition and selection. *Autophagy* 2015;11:1956-1977.
- 43 Kang X, Yang W, Feng D, Jin X, Ma Z, Qian Z, Xie T, Li H, Liu J, Wang R, Li F, Li D, Sun H, Wu S: Cartilage-Specific Autophagy Deficiency Promotes ER Stress and Impairs Chondrogenesis in PERK-ATF4-CHOP-Dependent Manner. *J Bone Miner Res* 2017;32:2128-2141.
- 44 B'chir W, Maurin AC, Carraro V, Averous J, Jousse C, Muranishi Y, Parry L, Stepien G, Fournoux P, Bruhat A: The eIF2 α /ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res* 2013;41:7683-7699.
- 45 Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkoul K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG: The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J Clin Invest* 2010;120:127-141.