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Anthocyanins from Chinese Bayberry Extract Activate Transcription Factor Nrf2 in β Cells and Negatively Regulate Oxidative Stress-Induced Autophagy

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Supporting Information

ABSTRACT: Islet replacement is a promising cure for insulin-dependent diabetes but is limited by a massive early cell death following transplantation. Overburden oxidative stress is one of the major factors causing cell damage. We have shown previously that anthocyanins in Chinese bayberry extract protected β cells (INS-1) from hydrogen peroxide (H_2O_2)-induced apoptosis and decreased grafts' apoptosis after transplantation partially through heme oxygenase-1 (HO-1) up-regulation. In the present study, we observed that H_2O_2 stimulation induced autophagy in β cells. Inhibition of autophagy increased cell viability and decreased cell death. Anthocyanin pretreatment attenuated oxidative stress-mediated autophagic cell death. Anthocyanins activated antioxidant transcription factor Nrf2 in INS-1 cells, and Nrf2/HO-1 negatively regulated autophagy process. Furthermore, we here demonstrate that autophagy also took place in β cell grafts during the early post-transplantation phase. β Cells pretreated with anthocyanins displayed decreased extent of autophagy after transplantation. Taken together, these findings further supported the conclusion that anthocyanins could serve as a protective agent of β cells and suggested that autophagy might play a role in β cells during transplantation.

KEYWORDS: *anthocyanins, Nrf2, heme oxygenase-1, islet transplantation, autophagy, oxidative stress*

INTRODUCTION

Islet transplantation is currently an effective treatment of type 1 diabetes that achieves insulin independence. However, the grafts will experience progressive deterioration of cellular viability and function overtime, leading to a large proportion of subjects returning to insulin dependence several years later.[1] This outcome is mainly attributed to (1) early graft damage during islet isolation, preservation, and early post-transplantation phase; during this stage, hypoxia, interrupted blood supply, ischemia/reperfusion injury, and nonspecific inflammation are the main causes of β cell death, with the highest percentage of islet graft loss and dysfunction occurring just days after transplantation; and (2) late phase loss, in which immunoreactions, which are capable of inflicting β cell damage and impairing β cell function, play major roles. Because of reduced antioxidant defenses, β cells are increasingly vulnerable to oxidative/nitrosative damage arising during these stages.[2] Increasing antioxidant capacity to render islets more resistant to free radical damage is theoretically a promising strategy to delay cell failure. Antioxidant treatment is reported to possess functions of decreasing free radical-induced β cell damage in several animal and human studies.[3]

Autophagy is the major cellular pathway for the degradation of long-lived proteins and cytoplasmic organelles in animal cells.[4] During autophagy, cytoplasmic materials are sequestered into double-membrane vesicles, "autophagosomes", which then fuse with lysosomes to form autolysosomes, where degradation of cellular structures occurs.[5,6] Autophagy could be stimulated by a change of environmental conditions such as nutrient deprivation. Hypoxia, ischemia/reperfusion, and excessive free radicals, which are also the stress stimulations during islet transplantation as mentioned above, are considered as the inducers of autophagy in certain circumstances. However, up to now, limited information has been reported about whether autophagy appears in β cells during transplantation procedure.

Chinese bayberry is one of six *Myrica* species native to China and possesses various biological activities including notable radical-scavenging ability.[7−9] The fruit extract is rich in anthocyanins, and cyanidin-3-*O*-glucoside (C3G) is identified as a major anthocyanin component.[9,10] We previously showed that anthocyanin pretreatment protected pancreatic β cells (rat insulinoma cell line INS-1) against hydrogen peroxide (H_2O_2)-induced necrosis and apoptosis via HO-1 induction and decreased graft apoptosis after transplantation.[11] In the present study, we explore the occurrence of autophagy in β cells during oxidative stress stimulation in vitro and at early post-transplantation phase and further investigate the protective

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effect of anthocyanins with the emphasis on their impact on autophagy.

MATERIALS AND METHODS

Chinese Bayberry Fruit Extract Preparation. Chinese bayberry fruits were obtained from Yuyao county, Zhejiang Province, China. The fruit was then ground and extracted with extract solvent (methanol acidified with 0.05% HCl) as described previously.[11] After being extracted three times and dried by evaporation at low temperature, the extract was dissolved in 80% ethanol solvent (4.5% formic acid) to remove impurities such as sugar. After being dried by evaporation again, anthocyanins were dissolved in double-distilled water and underwent further cryoconcentration. HPLC analysis of the extract was carried out,[10] and the content of C3G was calculated. HPLC analysis demonstrated that C3G accounted for 95% of total anthocyanins with a dominant peak at 520 nm. The concentration of C3G was 2.77 mmol/L (1.245 mg/mL), determined by peak area measurement with pure C3G as standard sample (Supporting Information, Figure S1). Because C3G is the major component, the usage dose of anthocyanins was standardized by the concentration of C3G.

Cell Culture Conditions, Reagents, and Plasmid. Rat pancreatic β cell line INS-1 was grown in RPMI-1640 supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere (5% CO_2 and 95% air). 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue dye, 3-methylamphetamine (3-MA), monodansylcadaverine (MDC, a specific autophagic vacuole marker), and Hoechst 33258 (H33258) were products of Sigma Chemical Co. (St. Louis, MO, USA). The commercially available in situ cell apoptosis detection kit (used for the TUNEL test) was purchased from Promega (Madison, WI, USA). Annexin V-FITC/PI apoptosis detection kit was obtained from Abcam Public Limited Co. (Abcam, Cambridge, MA, USA). Lipofectamine 2000 transfection reagent was obtained from Invitrogen (Carlsbad, CA, USA). GFP-mRFP-LC3 plasmid was kindly provided by Prof. T. Yoshimori (Osaka University, Osaka, Japan). The pLNCX2-HO-1 vector carrying the coding sequence of the rat HO-1 gene and INS-1 cells, which were transfected with pLNCX2-HO-1 stably overexpressing HO-1 (INS-1/HO1), were established in our previous study.[11]

Cell Viability Assay. Cell viability was determined using MTT dyes as previously described.[12] INS-1 cells (5×10^3 per well) were seeded in 96-well plates; after 1 mM H_2O_2 stimulation for 2 h, 20 μL of MTT solution was added to each well. After 4 h of incubation, the supernatant was removed and 150 μL of DMSO was added to each well. The plates were shaken for 10 min, and optical density was measured by an ELISA reader (BIO-Tek ELx800, Winooski, VT, USA) at a wavelength of 570 nm. Cell viability was also measured using the trypan blue dye exclusion test as previously described.[11] After trypan blue dye was added to treated INS-1 cells for 5 min, the number of stained cells (unviable cells) was counted and calculated as a percent of total number of cells.

Flow Cytometric Detection of Apoptosis. Cellular apoptosis was detected quantitatively by flow cytometry (FCM) as previously described.[12] After treatment, 1×10^6 INS-1 cells were suspended in 1 mL of 1× PBS and centrifuged (1200 rpm × 5 min), then the supernatant was removed, and cells were resuspended in 500 μL of 1× binding buffer. Ten microliters of annexin V-FITC and 5 μL of PI were added and kept in darkness with incubation for 15 min. Cellular apoptosis was assessed by FCM using the Cell Quest program (Becton Dickinson, San Jose, CA, USA).

Transmission Electron Microscopy (TEM). After experimental manipulations, cells or tissues were fixed in 2.5% glutaraldehyde and 1% osmic acid for 1 h, respectively, followed by 4% uranyl acetate for 30 min and gradient ethanol dehydration. Finally, the fixed samples were infiltrated with absolute acetone and the final resin mixture. Then the materials were embedded in Epon 812, cut with an Ultracut UCT ultramicrotome (Leica, Bensheim, Germany), and photographed by electron microscopy (JEM-1230).

Protein Extraction and Western Blot Analysis. Whole cell lysates were obtained by resuspending cells in a cell lysis buffer containing a protease inhibitor cocktail as previously described.[12] Nuclear and cytoplasmic extracts were prepared using commercial NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA; catalog no. 78833). Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology). Protein samples (equal amounts) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes, which were then blocked with 5% nonfat milk and probed overnight with primary antibody at 4 °C. After undergoing three washes, the membranes were incubated with secondary antibodies. The blots were visualized by enhanced chemiluminescence. Primary antibodies against beclin-1 (BECN1), Nrf2, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-LC3 antibody was obtained from MBL International (MBL, Nagoya, Japan). Antibody against HO-1 was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Caspase-3 and lamin A/C antibodies were obtained from Abcam Public Limited Co.

Electrophoretic Mobility Shift Assay (EMSA). To determine whether anthocyanins increase Nrf2 DNA-binding activity, we implemented EMSA using the Viagene EMSA kit (Viagene Biotech Inc., China) following the manufacturer's protocol. Briefly, equal amounts of nuclear protein (5 μg) were incubated with the biotin-labeled Nrf2 oligonucleotide and poly dI:dC for 20 min at room temperature in binding reaction buffer. The DNA–protein complexes were resolved on a 6.5% polyacrylamide gel pre-electrophoresed in 0.25× TBE solution at 120 V for 1 h. Then the gel was transferred onto a positively charged nylon membrane. The transferred DNA was cross-linked to the membrane and detected with horseradish peroxidase-conjugated streptavidin.

Monodansylcadaverine (MDC) Staining and Nrf2 Immunofluorescence. INS-1 cells were grown on glass coverslips. After being treated with the indicated agents, cells were incubated with 0.05 mM MDC for 20 min at 37 °C. Cells were washed two times with PBS and observed under fluorescence microscope.

For immunofluorescence detection of Nrf2, treated cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. After treatment with Triton X-100 (0.1%) for 10 min and normal goat serum for 30 min at room temperature, anti-Nrf2 antibody (Santa Cruz, CA, USA; 1:200 in 2% BSA) was added and incubated overnight at 4 °C. After an additional incubation for 1 h at room temperature with Alexor 488-conjugated goat anti-rabbit antibody (Molecular Probe, Invitrogen, Carlsbad, CA, USA) (1:200 in PBS), the slides were mounted and observed under fluorescence microscope.

Plasmid Transfection and RNA Interference. For detection of autophagosomes, INS-1 cells were transfected with GFP-mRFP-LC3 plasmid using Lipofectamine 2000 reagent, and the transfected cells were treated with H_2O_2 for 2 h and observed under fluorescence microscope. For RNA interference studies, the target sequence for HO-1-specific small interference RNA (siRNA) was 5′-CCGUGG-CAGUGGGAAUUUAUGCCAU-3′ and BECN1 siRNA sequence was 5′-GUCCCUGACAGACAAAUCU-3′, both of which and the control siRNA (no silencing) were synthesized by GenePharma Co. (Shanghai, China). Nrf2 siRNA was purchased from Santa Cruz Biotechnology (catalog no. sc-156128). Transfection was performed using Lipofectamine 2000 reagent according to the manual instructions.

Animal Studies. ICR mice of 8 weeks in age were used as the recipients in β cell transplantation experiment, and the experimental procedures were described in our previous study.[11] Pretreated INS-1 cells (2×10^6 cells with 1 μM anthocyanins for 24 h) and untreated INS-1 cells (2×10^6 cells) were transplanted under renal capsules of ICR mice, respectively. Seventy-two hours after transplantation, β cell grafts with a part of kidneys were harvested and fixed with 4% PFA, embedded in paraffin for immunohistological analysis. Immunohistological staining for LC3, BECN1, and TUNEL staining were performed according to the manual instructions. Some samples were fixed in glutaraldehyde for further TEM analysis as described above.

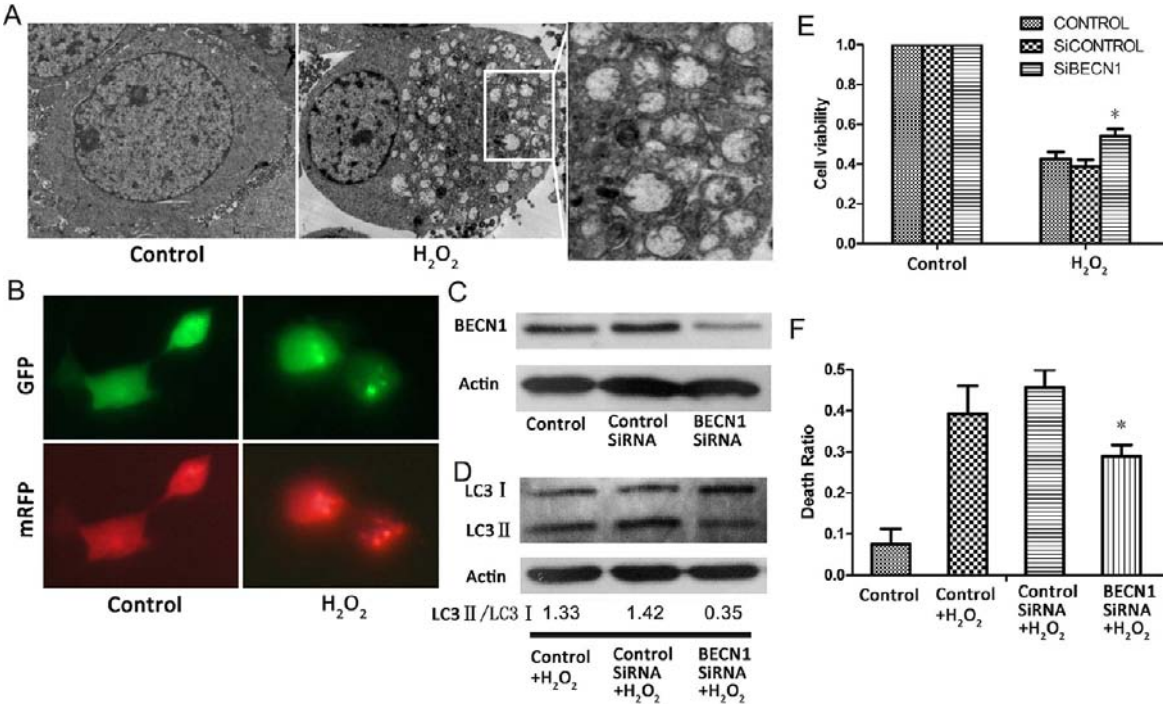

Figure 1. H_2O_2 stimulation induced autophagic cell death in β cells. (A) INS-1 cells were treated with 1 mM H_2O_2 for 2 h and underwent TEM examination. (B) Representative micrographs of INS-1 cells show the punctate dots of LC3 in the cytoplasm. Cells were transiently transfected with GFP-mRFP-LC3 plasmid for 24 h and treated with/without H_2O_2 for 2 h. (C) INS-1 cells were transfected with BECN1 siRNA or control siRNA (nonsilencing). BECN1 expression was analyzed by immunoblotting with anti-BECN1 antibody 48 h after transfection (nearly 70% down-regulation). (D) Cells transfected with BECN1 siRNA displayed decreased expression of LC3II after H_2O_2 treatment compared with control group. (E, F) After treatment of BECN1/control siRNA-transfected cells with 1 mM H_2O_2 for 2 h, cell viability was measured by MTT assay and trypan blue exclusion assay. (*) $p < 0.05$ versus control siRNA-transfected cells.

All experiments were carried out in accordance with the ethical guidelines of the Animal Experimentation Committee in our institute.

Statistical Analysis. When necessary, data were expressed as the mean ± SD. Statistical analysis was performed using SPSS version 13.0. One-way ANOVA with Bonferroni's post hoc test and Student's t test were used to evaluate statistical significance, and $p < 0.05$ was considered significant.

RESULTS

H_2O_2 Stimulation Induced Autophagic Cell Death in β Cells. We previously demonstrated that H_2O_2 injured INS-1 cells in a dose- and time-dependent manner, and we selected 1 mM H_2O_2 stimulation for 2 h as stress condition, which was also chosen for the present study. Because oxidative stress was also an autophagy inducer, we further explored whether autophagy was activated in H_2O_2-treated β cells. As shown in Figure1A, in comparison to control cells, extensive swelling of mitochondria and autophagic vacuolization could be observed in H_2O_2-treated cells under TEM. Microtubule-associated protein 1 light chain 3 (MAP LC3 or LC3) is a specific marker for autophagosome formation. The GFP-mRFP-LC3 vector, carrying the coding sequence of LC3 gene, was transfected into INS-1 cells, and cells with GFP-mRFP-LC3 vacuoles were observed under a fluorescent microscope. Figure1B clearly indicates that GFP-mRFP-LC3 is localized in vacuoles (dots) after 2 h of H_2O_2 treatment, whereas in control cells, LC3 fusion protein distributed in a diffuse pattern. These results supported the occurrence of autophagy in β cells under oxidative stress condition.

Because autophagy has two different effects (pro-survival or pro-death), we next investigated the role of autophagy in H_2O_2-induced cell death. The expression of BECN1 was down-regulated through siRNA transfection (about 70% down-regulation), leading to decreased extent of autophagy after H_2O_2 stimulation (Figure1C,D). We found that H_2O_2-induced cell death was mitigated after BECN1 down-regulation (Figure1E,F), and the mitigation was independent of apoptosis (Supporting Information, Figure S2), suggesting the pro-death role of H_2O_2-induced autophagy (in other words, "autophagic cell death").

Anthocyanins Treatment Attenuated H_2O_2-Induced Autophagic Cell Death and HO-1 Up-regulation Participated in This Process. Our previous study demonstrated that anthocyanins could time- and dose-dependently up-regulate HO-1 expression, which was involved in the protective mechanism of anthocyanins against H_2O_2-induced necrotic and apoptotic cell death. We here further investigate its impact on oxidative stress-induced autophagic cell death. As MDC accumulates in mature autophagic vacuoles, such as autophagolysosomes, MDC staining can be used to detect autophagic vacuoles. As shown in Figure 2A, compared with the H_2O_2-treated group, cells preincubated with anthocyanins displayed decreased accumulation of MDC. Immunoblotting analysis demonstrated decreased LC3II generation in the presence of 0.5 and 1 μM anthocyanins, compared with cells treated with H_2O_2 alone (Figure 2B).

To explore whether HO-1 was involved in H_2O_2-induced autophagic cell death, we first transfected β cells with HO-1 siRNA to knock down HO-1 expression (Figure 2C). Figure 2D shows that INS-1 cells, with reduced HO-1 expression, displayed much notable autophagic cell death, as reflected by increased LC3II production compared with control siRNA

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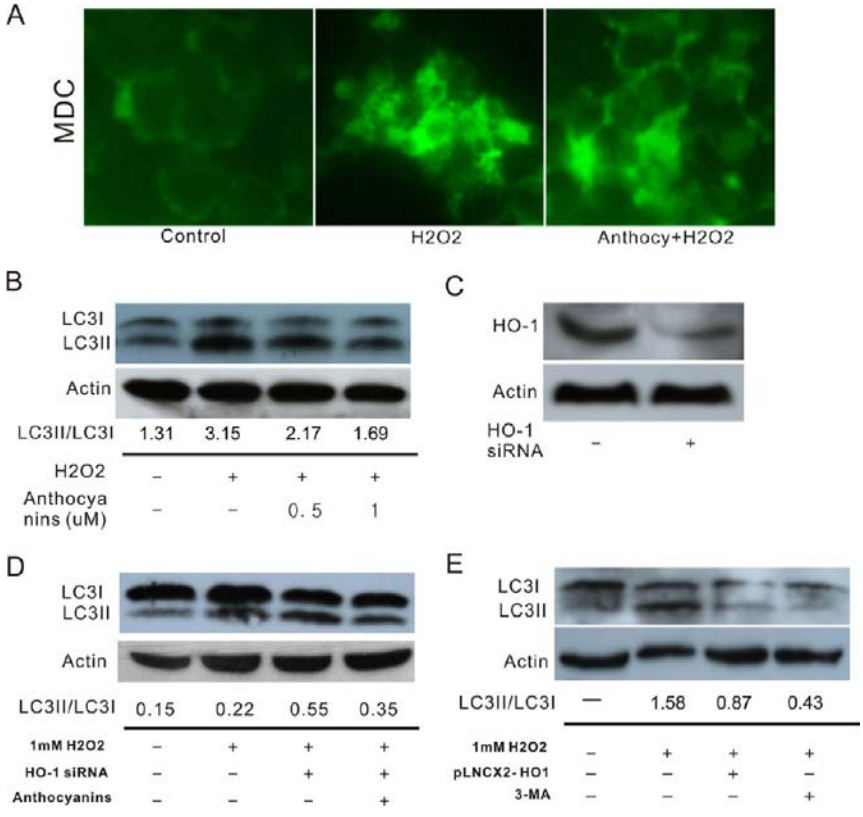

Figure 2. Anthocyanins attenuated H_2O_2-induced autophagic cell death and HO-1 was involved in regulation. (A) INS-1 cells pretreated with/without anthocyanins were incubated with 1 mM H_2O_2 for 2 h, stained with MDC (0.05 mM), and then visualized under a fluorescence microscope. (B) Immunoblotting analysis showed LC3-II expression in INS-1 cells following treatment with H_2O_2, in the absence or presence of anthocyanins preincubation. (C) INS-1 cells were transfected with control siRNA or HO-1 siRNA. Forty-eight hours later, the expression of HO-1 protein was determined. (D) INS-1 cells were transfected with HO-1/control siRNA, and 24−48 h later, transfected cells were then incubated with/without anthocyanins for another 24 h, followed by 1 mM H_2O_2 stimulation for 2 h. The expression of LC3II and the ratio of LC3II to LC3I were determined. (E) INS-1 cells stably overexpressed HO-1 (INS-1/HO1), and control cells were stimulated with 1 mM H_2O_2 for 2 h. Immunoblotting for LC3I/II was carried out. The 3-MA-pretreated (5 mM for 12 h) group served as reference.

transfection group, whereas in INS-1 cells constitutively overexpressing HO-1 protein (INS-1/HO1), H_2O_2-induced autophagy decreased (Figure 2E). In addition, HO-1 knockdown also attenuated anthocyanins' autophagy reduction effects, suggesting that anthocyanin-induced HO-1 expression participated in autophagy process.

Involvement of Nrf2 Activation in Anthocyanin-Induced HO-1 Up-regulation and Autophagy Process. Nrf2 is known as an important transcription factor involved in antioxidant response, binding to antioxidant response elements (ARE), and encoding detoxification enzymes including HO-1. We therefore examined whether Nrf2 is involved in anthocyanin-induced HO-1 expression. Immunofluorescence staining of Nrf2 showed that Nrf2 translocated from cytoplasm to nucleus in response to anthocyanin treatment (Figure 3A). Such translocation was further supported by immunoblotting analysis (Figure 3B). To evaluate anthocyanin-mediated Nrf2 transcription factor-binding activity in INS-1 cells, EMSA was performed. As shown in Figure 3C, treatment of cells with 1 or 2 μM anthocyanins for 12 and 24 h increased the DNA binding activity of Nrf2 in nuclear extracts. There was no detectable DNA binding complex without loading protein. To investigate whether anthocyanin-induced HO-1 expression is mediated through Nrf2 activation, cells were transfected with Nrf2 siRNA for 24 h followed by anthocyanin stimulation. Transfection of Nrf2 siRNA reduced Nrf2 expression (Figure 3D), as well as anthocyanin-induced HO-1 expression (Figure 3E). Furthermore, Nrf2 siRNA exacerbated H_2O_2-induced autophagy (Figure 3F), indicating that Nrf2/HO-1 plays a protective role under such circumstances.

Occurrence of Autophagy in β Cell Graft at the Early Post-transplantation Phase. To observe whether autophagy took place in β cell grafts, INS-1 cells were transplanted under the left renal subcapsule. Seventy-two hours later, the recipient was sacrificed and the graft was resected. As shown in Figure 4A,B, TEM displayed autophagic vacuolization in β cell grafts. Immunofluorescence staining for MAP LC3 also showed detectable punctate dots of LC3 in the cytoplasma (Figure 4C). These observations supported the occurrence of autophagy at the early phase of transplantation (72 h). In addition, TUNEL-positive staining verified that some cells underwent apoptosis (Figure 4D).

Anthocyanins Decreased the Extent of Autophagy in Grafts. We previously found that grafts pretreated with anthocyanins displayed less apoptosis, so we here focused on its impact on autophagy process. Figure 5 shows that there were extensive LC3 immunopositive dot-like structures in cytoplasma of transplanted INS-1 cells (Figure 5B), whereas the extent of punctuate dots of LC3 in grafts significantly decreased after anthocyanin preincubation (Figure 5A). The intensity of immunohistochemical staining for BECN1 in the anthocyanin-pretreated group was also less positive than that in

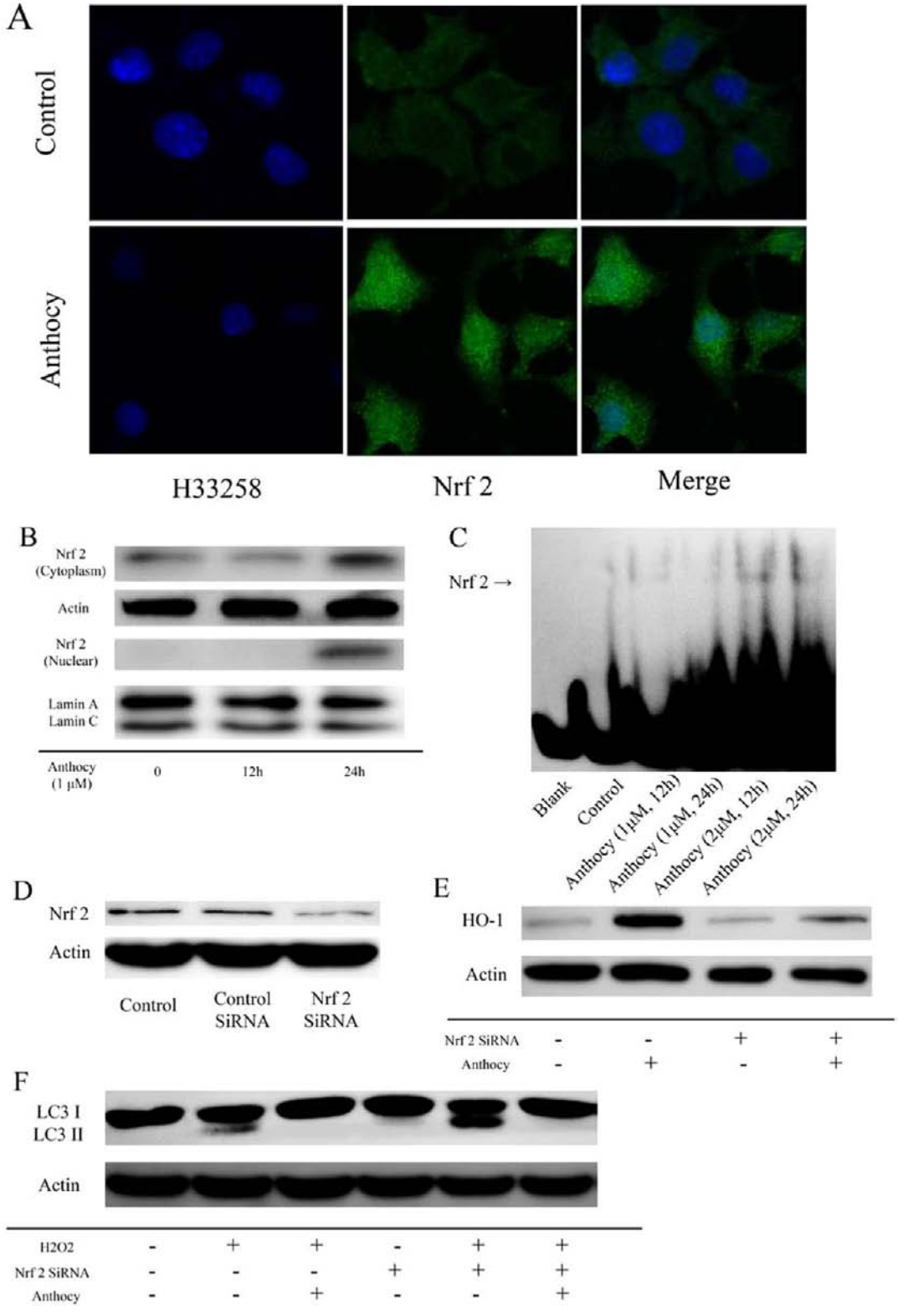

Figure 3. Involvement of Nrf2 activation in anthocyanin-induced HO-1 up-regulation and H_2O_2-induced autophagy. (A) After incubation with or without 1 μM anthocyanins for 24 h, cells were stained with anti-Nrf2 antibody and nuclei with H33258 dye. (B) INS-1 cells were treated with 1 μM anthocyanins for 12 or 24 h, and then the protein expression of Nrf2 in cytoplasmic and nuclear extracts was determined by immunoblotting. (C) After stimulation with or without anthocyanins (1 or 2 μM) for 12 and 24 h, the nuclear extracts of cells were obtained and EMSA was performed to monitor Nrf2-ARE binding activity in the nuclear fraction. (D) INS-1 cells were transfected with Nrf2 siRNA; 48 h later, the Nrf2 expression was determined. (E) Cells were transfected with Nrf2 siRNA for 24 h, followed by stimulation with 1 μM anthocyanins for another 24 h. Then, the protein level of HO-1 was observed by immunoblotting. (F) After transfection with or without Nrf2 siRNA for 24 h, followed by 1 μM anthocyanins for 24 h, INS-1 cells were then stimulated with 1 mM H_2O_2 for 2 h. The expression of LC3I/II protein was determined. Note that Nrf2 down-regulation exacerbated H_2O_2-induced autophagic cell death.

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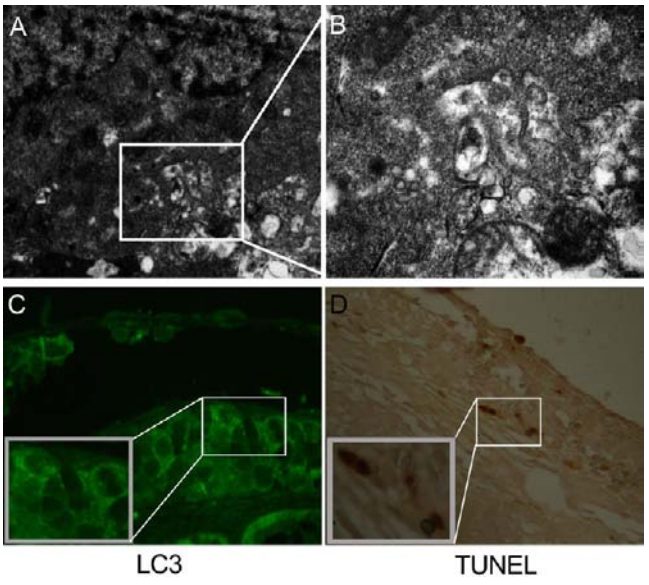

Figure 4. Autophagy occurred in β cell graft at the early phase of post-transplantation. INS-1 cells were transplanted under renal capsule of mice. and 72 h later, the grafts were removed. (A, B) TEM examination displayed the presence of autophagosomes and autolysosomes in graft cells. (C) Punctate LC3 dots were observed in grafts detected by immunofluorescent for MAP LC3. (D) A portion of cells undergoing apoptosis were TUNEL positive.

control cells (Figure 5C,D). Together with our previous observations, these results suggest that treating grafts with anthocyanins prior to transplantation could decrease cellular death at early phase of post-transplantation through down-regulating both apoptotic and autophagic processes.

DISCUSSION

This work is a further step of our previous study; we here show that (1) anthocyanins protect INS-1 cells from autophagic cell death induced by exogenous H_2O_2 stimulation, (2) autophagy happens in β cell grafts at early post-transplantation phase and anthocyanin pretreatment decreases the extent of autophagy, and (3) antioxidant enzyme expression may be associated with autophagy process. These findings, combined with our previous results, further support that anthocyanins in Chinese bayberry extract may serve as an islet-protective agent.

Autophagy plays an important role in cellular metabolism and is usually activated under stress conditions such as starvation, ischemia/reperfusion, pathogen infection, and certain chemical stimulus. Recent studies showed that oxidative stress could elicit increased autophagy.[13−15] Exogenous H_2O_2 treatment leads to oxidative stress and mitochondrial damage, which induces autophagy. It is also reported that H_2O_2 treatment can mimic tumor necrosis factor α-induced autophagy and trigger cell death.[16] In the present study, exogenous H_2O_2 stimulation was applied to increase intracellular ROS production in β cells, and we also observed the activation of autophagy in pancreatic β cells after H_2O_2 treatment. H_2O_2 caused excessive intracellular ROS production[11] and mitochondrial damage (indicated by TEM) and activated autophagy and apoptosis simultaneously. Further research showed that down-regulating BECN1 expression by siRNA attenuated H_2O_2-induced cell injury (which was independent of apoptotic process), suggesting its pro-death role under this circumstance. Such a conclusion was contrary to the previous study displaying autophagy acted as a survival pathway in a similar situation.[17] We suspected that the different effects of autophagy might be attributed to different cell types, considering the susceptibility of β cells to ROS.

In recent years, there have been increasing studies performed to investigate the role of autophagy in β cells and diabetes.[18] Altered autophagy was observed in β cells of Zucker diabetic

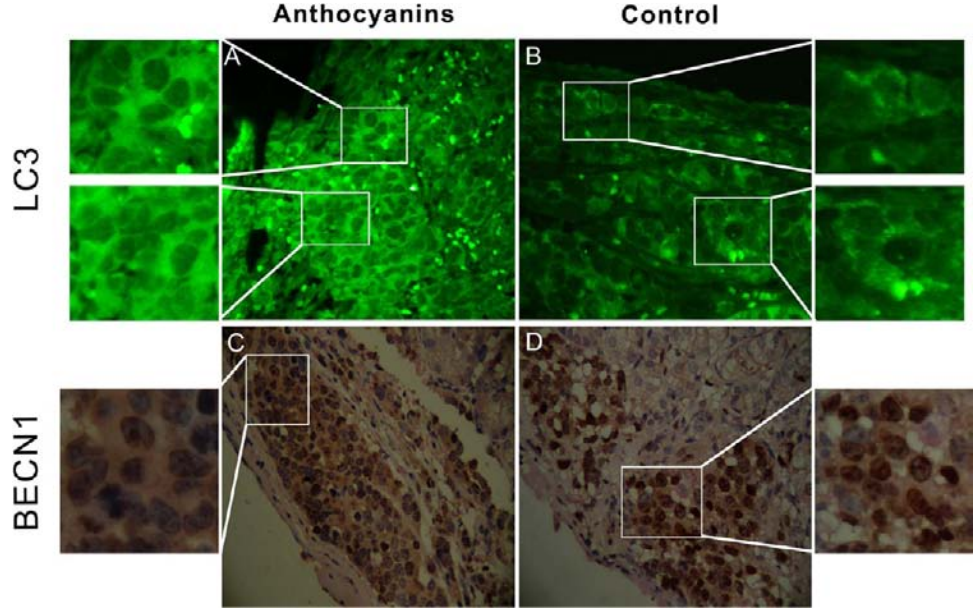

Figure 5. Anthocyanin pretreatment reduced the autophagy occurring in β cells at the early phase of post-transplantation. (A−D) Immunochemical detection of LC3 and BECN1: INS-1 cells pretreated with/without anthocyanins for 24 h were transplanted under renal capsule, and 72 h later, the grafts were removed. Representative sections demonstrated that graft cells pretreated with anthocyanins displayed fewer LC3 dots in cytoplasma (A) and were less immunopositive for BECN1 (C) compared with control cells (B, D).

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fatty rats, diabetic db/db mice, high-fat-fed C57BL/6 mice, and also in β cell lines following high-glucose or palmitate treatment.[19−22] An unbalanced autophagy process was considered to be associated with β cell dysfunction. Masini et al.[23] observed that β cells in patients with type 2 diabetes have signs of altered autophagy, which might contribute to the loss of β cell mass. In the field of islet transplantation, the early occurrence of apoptosis and necrosis in islet grafts was confirmed in previous studies, but the questions of whether autophagy participates in this process and its role in islet dysfunction have not yet been investigated. We here, for the first time, observed that autophagy also occurred at early post-transplantation phase (72 h) in subrenal capsular transplantation model, supported by the TEM and MAP LC3 immunostaining. Anthocyanins in CBE protected β cells against H_2O_2-induced autophagic cell death in vitro, and pretreating with anthocyanins also reduced autophagosome accumulation in β cell transplantation model. In this context, although the role of autophagy in the fate of β cell grafts (pro-survival or pro-death) was not determined here, combined with our previous and present results, the decreased extent of both apoptosis and autophagy reflected the improved endurance of INS-1 cells to the hypoxia and ischemic stress in the graft's microenvironment, resulting in the survival of more viable and functional cells.

To exclude the possibility of direct scavenging H_2O_2 by anthocyanins, the culture medium containing anthocyanins, which was used to pretreat INS-1 cells, was removed before H_2O_2 stimulation in this study. We found that anthocyanins increased transcriptive activity of Nrf2, which was associated with the up-regulation of HO-1. It is well established that the Nrf2−Keap1 pathway functions as a critical regulator of the cell's defense mechanism against oxidative stress by inducing the expression of many cellular protective proteins.[24] We here observed that HO-1 overexpression (via gene transfection or anthocyanins inducing) rescued cells from oxidative stress-induced autophagy. Knockdown of HO-1 or Nrf2 exacerbated H_2O_2-induced autophagy, suggesting that Nrf2/HO-1 pathways are involved in regulating (oxidative stress-mediated) autophagy in β cells. These results were, to some extent, consistent with a previous study, in which Rao et al.[25] observed Nrf2-regulated enzyme NQO1 played an essential role in controlling the level of autophagy and knockdown of Nrf2 caused an increase in autophagy. Similarly, HO-1 was reported to protect against cigarette smoke extract-induced cell death by concurrently down-regulating apoptosis and autophagy-related signaling.[26] Bolisetty et al.[27] observed that HO-1-deficient cells displayed higher levels of basal autophagy, whereas overexpression of HO-1 decreased the extent of cisplatin-induced cell autophagy.

Overexpression of antioxidant enzymes, in most situations, could reduce the occurrence of autophagy as mentioned above. However, the detailed regulation process has not been fully determined yet. It is reported that HO-1 expression could change the autophagic level via directly modulating the association of Beclin-1 with Bcl-xL and Rubicon, a novel negative regulator of autophagy.[28] Other research displayed that a direct interaction existed between the Nrf2−Keap1 pathway and p62,[29] a crucial adaptor between LC3-decorated autophagosomes and ubiquitin-conjugated protein aggregates. Keap1, a negative controller of Nrf2 activation, could directly regulate autophagy through its interaction with LC3 and p62.[30−32] For the present study, whether up-regulated Nrf2 itself or its induced HO-1 overexpression controlled the autophagic process is largely unknown. Furthermore, the significance of interactions among oxidative stress, Nrf2−Keap1 pathway, autophagy (p62) in β cells (including transplantation), and diabetes remains to be further elucidated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

C3G, cyanidin-3-*O*-glucoside; CBE, Chinese bayberry extracts; MAP LC3, microtubule-associated protein 1 light chain 3; MDC, monodansylcadaverine; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ROS, reactive oxygen species; siRNA, small interference RNA; TEM, transmission electron microscopy

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