Mitochondrial Complexes I and II Are More Susceptible to Autophagy Deficiency in Mouse β-Cells

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Background: Damaged mitochondria are removed by autophagy. Therefore, impairment of autophagy induces the accumulation of damaged mitochondria and mitochondrial dysfunction in most mammalian cells. Here, we investigated mitochondrial function and the expression of mitochondrial complexes in autophagy-related 7 (*Atg7*)-deficient β-cells.

Methods: To evaluate the effect of autophagy deficiency on mitochondrial function in pancreatic β-cells, we isolated islets from *Atg7*[F/F]:RIP-*Cre*+ mice and wild-type littermates. Oxygen consumption rate and intracellular adenosine 5′-triphosphate (ATP) content were measured. The expression of mitochondrial complex genes in *Atg7*-deficient islets and in β-TC6 cells transfected with si*Atg7* was measured by quantitative real-time polymerase chain reaction.

Results: Baseline oxygen consumption rate of *Atg7*-deficient islets was significantly lower than that of control islets ($P<0.05$). Intracellular ATP content of *Atg7*-deficient islets during glucose stimulation was also significantly lower than that of control islets ($P<0.05$). By Oxygraph-2k analysis, mitochondrial respiration in *Atg7*-deficient islets was significantly decreased overall, although state 3 respiration and responses to antimycin A were unaffected. The mRNA levels of mitochondrial complexes I, II, III, and V in *Atg7*-deficient islets were significantly lower than in control islets ($P<0.05$). Down-regulation of *Atg7* in β-TC6 cells also reduced the expression of complexes I and II, with marginal significance ($P<0.1$).

Conclusion: Impairment of autophagy in pancreatic β-cells suppressed the expression of some mitochondrial respiratory complexes, and may contribute to mitochondrial dysfunction. Among the complexes, I and II seem to be most vulnerable to autophagy deficiency.

Keywords: Autophagy; Insulin-secreting cells; Mitochondria; Mitochondrial complex

INTRODUCTION

Autophagy is a cellular degradation-recycling system for aggregated proteins and damaged organelles. Autophagy to remove damaged mitochondria is termed mitophagy. Impairment of autophagy is known to induce the accumulation of damaged mitochondria and to cause mitochondrial dysfunction in most mammalian cells. The accumulation of deformed mitochondria has been noted in hepatocytes and cardiomyocytes of autophagy-deficient mice [1,2]. Autophagy-related 7 (*Atg7*), an E1-like

gene, is essential for the formation of autophagosomes [3], and *Atg7*-deficient erythrocytes show altered mitochondrial membrane potential [4]. This suggests that mitophagy plays a pivotal role in the control of mitochondrial quality and quantity [5].

Recently, autophagy has also been implicated in the regulation of pancreatic β-cell function and mass [6]. Mice with β-cell-specific deletion of *Atg7* showed impaired glucose tolerance and decreased serum insulin levels [7,8]. In those studies, electron microscopy of the autophagy-deficient β-cells revealed mitochondrial swelling. Other groups have reported decreased oxygen consumption rate and adenosine 5′-triphosphate (ATP) production in *Atg7*-deficient islets *ex vivo*, suggesting mitochondrial dysfunction [8,9]. In addition, reduced Ca^{2+} transients in response to glucose in autophagy-deficient β-cells have been noted [7], which may indicate abnormal mitochondrial function. Mitochondria are crucial in the generation of ATP, affecting both the closure of ATP-sensitive K^+ channels and Ca^{2+} influx. Here, we further investigated changes in *Atg7*-deficient β-cells, focusing on the level of the mitochondrial respiratory chain complexes.

METHODS

Mice and islet isolation

To generate mice with β-cell-specific deletion of *Atg7* (*Atg7*$^{Δβ-cell}$), *Atg7*-floxed (*Atg7*$^{F/F}$) mice were crossed with RIP-*Cre* mice. RIP-Cre mice or *Atg7*$^{F/F}$ mice were used as controls. After overnight fasting, pancreatic islets were isolated from 20-week-old mice using a collagenase digestion technique [7]. All animal experiments were conducted in accordance with the Institutional Guide for the Care and Use of Laboratory Animals of Seoul National University Hospital.

Cells and *Atg7* RNA interference

β-TC6 cells were obtained from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (GIBCO), in a humidified atmosphere of 95% air and 5% CO_2. To generate *Atg7*-deficient cells, β-TC6 cells were transfected with si*Atg7* by electroporation using an Amaxa Cell Line Nucleofector Kit V system (Lonza, Basel, Switzerland). The glucose concentration of the culture medium was changed to 15 mM during the 48 hours of transfection. Down-regulation of *Atg7* was verified by quantitative real-time polymerase chain reaction (RT-PCR).

Oxygen consumption rate

Baseline oxygen consumption rate was measured from 200 islets over 2 hours, using a BD Oxygen Biosensor System, according to the manufacturer's protocol (Becton Dickinson, San Jose, CA, USA). An Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) was used to examine the responses to mitochondrial effectors of 1,000 islets suspended in Krebs-Ringer bicarbonate HEPES (KRBH) buffer with 10 mM glucose, at 37°C. During the experiments, substances were added serially as described [10,11]: glutamate (10 mM) and malate (2 mM) (G+M) were first added as substrates for mitochondrial respiration; adenosine diphosphate (ADP, 2.5 mM) was then added to induce state 3 respiration; cytochrome c (10 μM) was added to test the integrity of the outer mitochondrial membrane; succinate (10 mM) was added for electron transfer to complex II; next, 0.5 to 1.5 μM carbonyl-cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), a mitochondrial respiration uncoupler, was added to obtain maximal oxygen consumption rate; rotenone (0.5 μM) was added to inhibit complex I, and then antimycin A (1.5 μM) to inhibit complex III.

Intracellular ATP content

Ten isolated islets in each of five culture wells were treated with low glucose-KRBH buffer (1.6 mM) or high glucose-KRBH buffer (16 mM) for 1 hour, after 1 hour of fasting. ATP content of the islets was then measured, using a bioluminescence assay kit (Sigma-Aldrich, St. Louis, MO, USA), and the values were normalized to total cellular protein in each sample. Protein concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

Real-time quantitative reverse transcription polymerase chain reaction of mitochondrial complex mRNAs

We isolated total RNA from mouse islets or β-TC6 cells using TRizol reagent (Ambion, Foster City, CA, USA), synthesized cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA), and amplified it using the SYBR Premix Ex Taq polymerase (TaKaRa, Otsu, Japan). Quantitative RT-PCR was performed and analyzed using a LightCycler 96 Real-Time PCR System (Roche, Mannheim, Germany). Primer sequences are presented in Table 1.

Statistical analysis

Results are presented as means±standard error. Statistical analyses of differences between groups were performed using

Student *t* test or the Mann-Whitney *U* test, depending on sample variances. *P* values <0.05 were considered to indicate statistical significance.

RESULTS

Basal oxygen consumption rate

To investigate the effects of autophagy on mitochondrial function, islets were isolated from $Atg7^{\Delta\beta\text{-cell}}$ and control mice, and oxygen consumption rate was measured *ex vivo* for 2 hours (Fig. 1). Oxygen consumption rate during that time increased 3-fold over initial values in control islets, while that of the $Atg7^{\Delta\beta\text{-cell}}$ islets changed nonsignificantly. This difference between the groups in fold change after 2 hours was significant (*P*<0.05).

Intracellular ATP content

We measured intracellular ATP content *ex vivo* in response to

glucose stimulation. The intracellular ATP content of islets from $Atg7^{\Delta\beta\text{-cell}}$ mice was significantly lower than that from
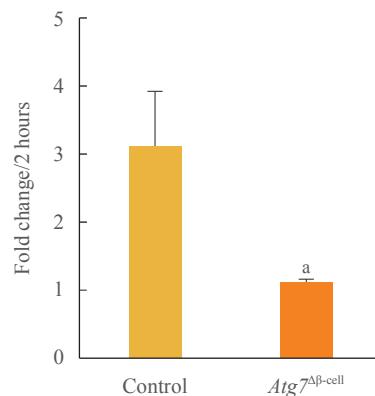

Fig. 1. Basal oxygen consumption rates in islets isolated from $Atg7^{\Delta\beta\text{-cell}}$ and control mice (*n*=5 to 8 per group). Data are presented as fold changes±SE, and comparisons were performed using a Mann-Whitney *U* test. *Atg7*, autophagy-related 7. [a]*P*<0.05 compared to the control.

Table 1. Sequences of Primers for Quantitative Real-Time Polymerase Chain Reaction

|	Forward	Reverse
ATG7	TCT GGG AAG CCA TAA AGT CAG G	GCG AAG GTC AGG AGC AGA A
Complex I (20 kDa)	CCA GCT GCG CAG AGT TCA TC	GAG AGA GCT TGG GGA CCA CG
Complex II (Ip)	TCT ACC GCT GCC ACA CCA TC	AAG CCA ATG CTC GCT TCT CC
Complex III (Core II)	CCA TTG GAA ATG CAG AGG CA	GGC TGG TGA CTT CCT TTG GC
Complex IV (COX2)	TCA TGA GCA GTC CCC TCC CT	GCC ATA GAA TAA CCC TGG TCG G
Complex V (F1α)	ATC TAT GCG GGT GTA CGG GG	AGG GAC TGG TGC TGG CTG AT
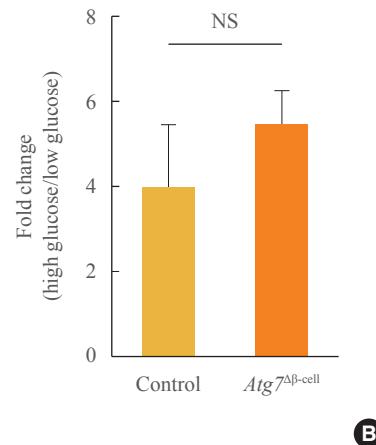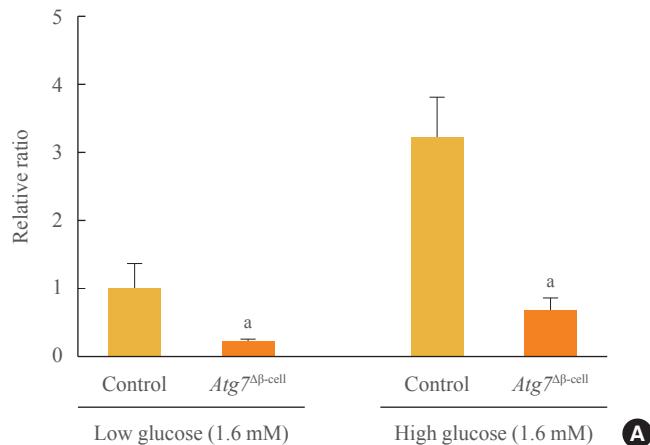
Fig. 2. Intracellular adenosine 5′-triphosphate (ATP) content induced by glucose stimulation of islets isolated from $Atg7^{\Delta\beta\text{-cell}}$ and control mice (*n*=3 to 10 per group). (A) ATP content under low-glucose (1.6 mM) and high-glucose (16 mM) conditions. All values were normalized relative to those of control islets under low glucose conditions. (B) Fold change in ATP content induced by high-glucose stimulation. Data are presented as fold changes±SE, and comparisons were performed using a Mann-Whitney *U* test. *Atg7*, autophagy-related 7; NS, not significant. [a]*P*<0.05 compared to control.
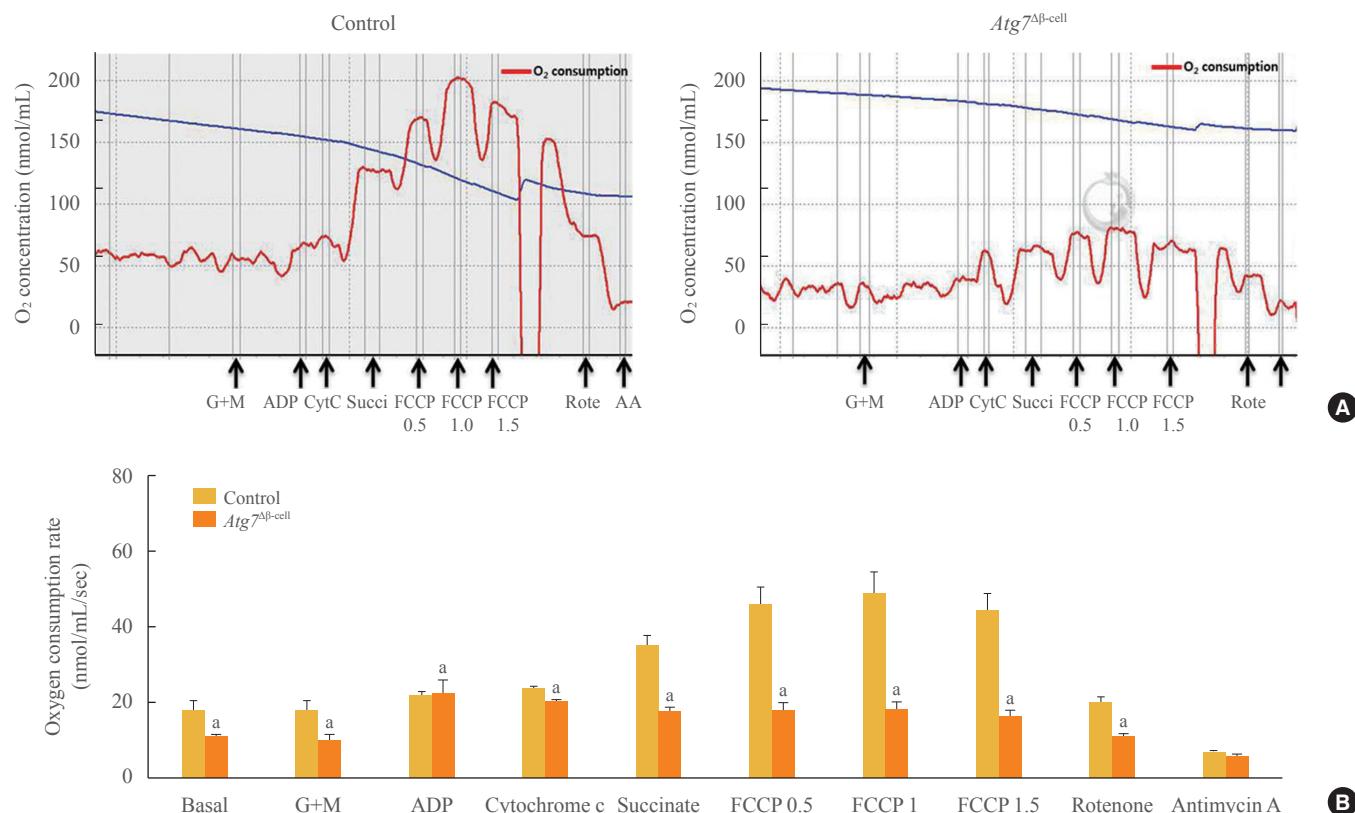
Fig. 3. Mitochondrial respiration in islets isolated from $Atg7^{\Delta\beta\text{-cell}}$ and control mice ($n=9$ to 10 per group). (A) Representative graphs of mitochondrial respiration using Oxygraph-2k. Red lines indicate oxygen consumption rate in response to sequential loading of mitochondrial effectors (indicated by arrows below the graphs). (B) Quantitative comparisons of oxygen consumption rate in response to effectors. Data are presented as means±SE, and comparisons were performed using repeated measures analysis of variance and Mann-Whitney U tests. G+M, glutamate and malate; ADP, adenosine diphosphate; FCCP, carbonyl-cyanide-4-(trifluoromethoxy)-phenylhydrazone; AA, antimycin A; $Atg7$, autophagy-related 7. [a]$P<0.05$ compared to control islets.

control mice, under both low- and high-glucose conditions ($P<0.05$) (Fig. 2A). However, the fold change in ATP production with high glucose was comparable between the two groups (Fig. 2B). This may be due to suppressed ATP production under both low- and high-glucose concentrations.

Mitochondrial respiration

It is unclear whether mitochondrial dysfunction reflects only the accumulation of damaged mitochondria or if there are other, direct effects on mitochondrial function. To determine whether specific respiratory states are influenced, we performed Oxygraph-2k analyses with isolated islets. Representative graphs are presented in Fig. 3A. Throughout the experiment, a marked reduction in oxygen consumption rate was observed in $Atg7^{\Delta\beta\text{-cell}}$ islets compared to controls ($P<0.01$ by repeated measures analysis of variance) (Fig. 3B). Inhibitory effects of succinate (an inhibitor of complex II) and rotenone (an

inhibitor of complex I) were relatively prominent, while there was no difference in responses to ADP (stage 3 respiration) or to antimycin A (an inhibitor of complex III). These findings suggest that complexes I and II are most vulnerable to autophagy deficiency.

Expression of mitochondrial complex genes

We examined the expression of mitochondrial respiratory complex genes in isolated islets and in β-TC6 cells. In $Atg7^{\Delta\beta\text{-cell}}$ islets, expression of $Atg7$ mRNA was reduced by half (Fig. 4A). Among mitochondrial complexes, mitochondrial complex IV gene expression predominated. The expressions of mitochondrial complexes except complex IV were significantly lower in $Atg7^{\Delta\beta\text{-cell}}$ islets than in controls ($P<0.05$) (Fig. 4A). In β-TC6 cells transfected with si$Atg7$ for 48 hours, $Atg7$ expression was also reduced by half ($P<0.05$); the expression of mitochondrial complexes I and II was reduced, but the sta-
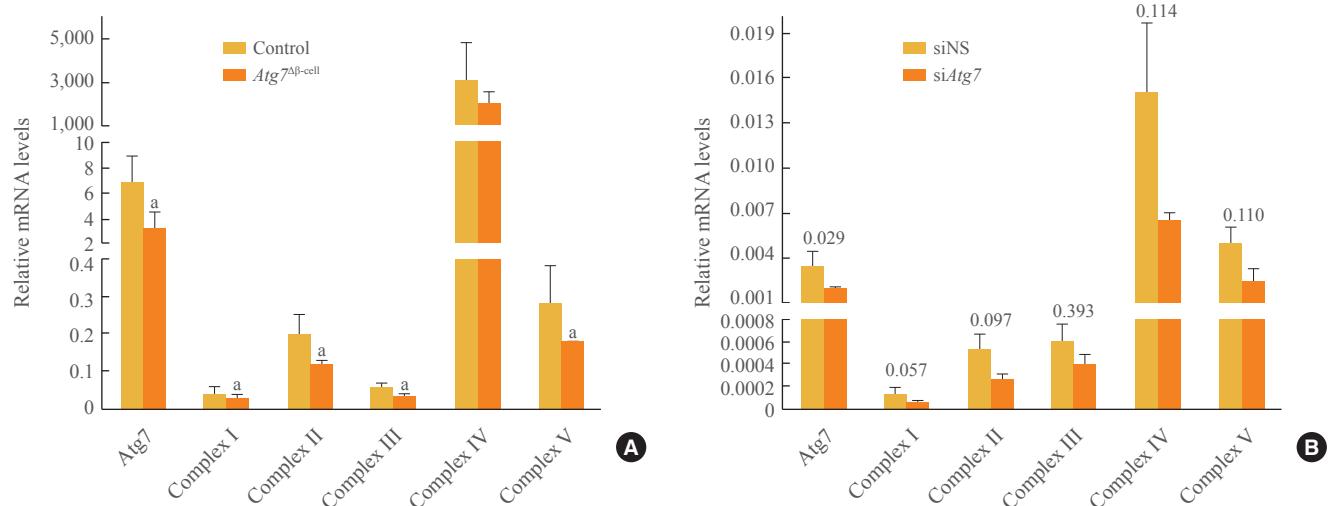
Fig. 4. Expression of mitochondrial complex genes by quantitative real-time polymerase chain reaction (A) in $Atg7^{\Delta\beta\text{-cell}}$ and control islets ($n=7$ to 8 per group), and (B) in β-TC6 cells transfected with si$Atg7$ or with siNS as a control ($n=4$). The expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase. Data are presented as means±SE, and comparisons were performed using Student t tests or Mann-Whitney U tests, depending on sample variances. P value is presented above the bar graph. $Atg7$, autophagy-related 7. [a]$P<0.05$ compared to control islets.

tistical significance of this effect was marginal (Fig. 4B).

DISCUSSION

In pancreatic β-cells, as in other mammalian cells, damaged mitochondria are removed by autophagy [12]. Impairment of autophagy is thus expected to result in the accumulation of damaged mitochondria, and we have previously reported abnormal swollen mitochondria in $Atg7^{\Delta\beta\text{-cell}}$ mice [7]. In this study, we observed the decreased oxygen consumption rate and ATP content, noted previously in autophagy-deficient pancreatic β-cells [8,9]. We also report that impaired autophagy affects specific steps in the mitochondrial electron transfer system. Although $Atg7^{\Delta\beta\text{-cell}}$ islets showed a significant overall decrease in mitochondrial respiration compared to control islets (Fig. 3A), state 3 respiration estimated by treatment with ADP was unaffected by autophagy deficiency, and treatment with antimycin A, an inhibitor of complex III, abolished the difference in oxygen consumption rate between $Atg7^{\Delta\beta\text{-cell}}$ islets and controls (Fig. 3B). Given the results of the significant differences by succinate and rotenone treatments, mitochondrial complexes I and II seemed to be most vulnerable to autophagy deficiency. Wu et al. [9] reported that $Atg7$-deficient mouse islets showed decreased mitochondrial respiration when treated with oligomycin, an inhibitor of complex V (ATP synthase), or with FCCP.

We evaluated mRNA expression of genes for components of each mitochondrial complex, both in mouse islets and in β-TC6 cells. Although Wu et al. [9] reported no difference in expression of these genes in $Atg7$-deficient skeletal muscle, we found the expression of all but one complex IV component gene to be decreased significantly in $Atg7^{\Delta\beta\text{-cell}}$ islets (Fig. 4A). Inhibition of $Atg7$ in β-TC6 cells *in vitro* also reduced mitochondrial complex expression, although this effect was generally not statistically significant (Fig. 4B). The reduction in mitochondrial complexes I and II was marginally significant ($P=0.057$ and $P=0.097$, respectively). We presume that autophagy inhibition initially affects the expression of complexes I and II, and that prolonged suppression of autophagy influences expression of complexes III and V, but not IV. Such differences in gene expression would contribute to differences in the functions of the complexes.

Although we did not investigate reactive oxygen species (ROS), it may also contribute to mitochondrial dysfunction in autophagy-deficient β-cells. Mitochondria are the primary organelle producing ROS, and are also significantly influenced by ROS. As inhibition of $Atg7$ has been reported to increase the generation of ROS in pancreatic β-cells, and the attenuation of ROS recovered cellular function [9], mitochondrial dysfunction in autophagy-deficient β-cells may be closely related to ROS generation. In this study, we determined that autophagy impaired mitochondrial function. On the other hand, mitochon-

drial damage can generate ROS, leading to autophagy induction [13]. Rotenone, an inhibitor of complex I, and theonoyltrifluoroacetone (TTFA), an inhibitor of complex II, induced autophagic cell death, and inhibition of *Atg5* ameliorated rotenone- or TTFA-induced cell death in transformed and cancer cell lines [14].

In conclusion, impairment of autophagy in β-cells *in vivo* induced mitochondrial dysfunction, particularly of mitochondrial respiratory complexes I and II. This suggests that not only the simple accumulation of damaged mitochondria but also direct effects on mitochondria, including altered expression of respiratory complex genes, may cause disturbed energy homeostasis by autophagy deficiency.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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