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Glucagon regulates hepatic mitochondrial function and biogenesis through FOXO1

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Abstract

Glucagon promotes hepatic glucose production maintaining glucose homeostasis in the fasting state. Glucagon maintains at high level in both diabetic animals and human, contributing to hyperglycemia. Mitochondria, a major place for glucose oxidation, are dysfunctional in diabetic condition. However, whether hepatic mitochondrial function can be affected by glucagon remains unknown. Recently, we reported that FOXO1 is an important mediator in glucagon signaling in control of glucose homeostasis. In this study, we further assessed the role of FOXO1 in the action of glucagon in the regulation of hepatic mitochondrial function. We found that glucagon decreased the heme production in a FOXO1-dependent manner, suppressed heme-dependent complex III (UQCRC1) and complex IV (MT-CO1) and inhibited hepatic mitochondrial function. However, the suppression of mitochondrial function by glucagon was largely rescued by deleting the *Foxo1* gene in hepatocytes. Glucagon tends to reduce hepatic mitochondrial biogenesis by attenuating the expression of NRF1, TFAM and MFN2, which is mediated by FOXO1. In *db/db* mice, we found that hepatic mitochondrial function was suppressed and expression levels of UQCRC1, MT-CO1, NRF1 and TFAM were downregulated in the liver. These findings suggest that hepatic mitochondrial function can be impaired when hyperglucagonemia occurs in the patients with diabetes mellitus, resulting in organ failure.

Key Words

- ▶ glucagon
- ▶ mitochondrial function
- ▶ biogenesis
- ▶ FOXO1

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Introduction

Glucagon secreted from the α -cells of pancreatic islets is a predominant counter regulatory hormone to the action of insulin in control of blood glucose homeostasis (Habegger *et al.* 2010). This 29 amino acid peptide hormone is generated from a proglucagon peptide through posttranslational processing (Baggio *et al.* 2007). Glucagon plays its primary function in the liver to maintain euglycemia during the fasting state, by promoting hepatic glucose output via increasing glycogenolysis and gluconeogenesis (Jiang *et al.* 2003). However, the glucagon concentration

or the ratio of glucagon to insulin is elevated in both type I and II diabetes mellitus, including animals and humans (Unger *et al.* 1978). Chronic hyperglucagonemia results in excess hepatic glucose production, enhancing blood glucose in patients with type 2 diabetes (T2D) (Consoli 1992). Inactivation of glucagon receptor is associated with significant improvement of blood glucose in diabetic mice (Lee *et al.* 2011). Therefore, glucagon is important in control of glucose metabolism in both healthy and pathological conditions.

FOXO1 is a member of the O class of forkhead/winged helix transcription factors and plays an important role in multiple metabolic actions including glucose and lipid metabolism (Zhang *et al.* 2006, Lee *et al.* 2017). Our early study demonstrated that FOXO1 is a substrate for AKT in the insulin signaling cascade, and FOXO1 is phosphorylated at T24, S256 and S319 by AKT. The phosphorylation of FOXO1 by AKT in insulin signaling cascades leads to ubiquitin-mediated FOXO1 degradation (Rena *et al.* 1999). Subsequently, expression levels of FOXO1 target genes, such as *G6pc* and *Pck*, are suppressed and hepatic glucose production inhibited (Nakae *et al.* 1999, Schmoll *et al.* 2000). During the fasting period, expression levels of FOXO1 target genes increase for promotion of gluconeogenesis, maintaining a normal blood glucose (Lu *et al.* 2012). Inactivation of FOXO1 in liver reduces blood glucose (Montgomery *et al.* 2015).

Mitochondrial dysfunction has been implicated in the development of insulin resistance and it is reported that FOXO1 stimulates gene expression of heme-oxygenase 1 (*Hmox1*), a key enzyme controlling the mitochondrial biogenesis and function. Moreover, a constitutively active FOXO1 disrupts hepatic mitochondrial function and biogenesis in T2D, suggesting that FOXO1 is also a key factor integrating insulin signaling and mitochondrial function in the liver (Cheng *et al.* 2009). Recently, we have just demonstrated that FOXO1 is a mediator in the glucagon signaling in control of glucose homeostasis (Wu *et al.* 2018). In this study, we further examined whether glucagon regulates mitochondrial function involving in FOXO1 in liver.

Experimental procedures

Animals

Liver-specific *Foxo1*-knockout mice (L-F1KO) were previously described in a mixed genetic background with C57B6/J and 129 (Zhang *et al.* 2012). The *db/db* mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed with ad libitum chow diet and all animal procedures were performed at Texas A&M University and approved by the Texas A&M Health Science Center Institutional Animal Care and Use Committee.

Real-time PCR analysis of gene expression

RNA was extracted from livers in 18-h fasted or random-fed male mice, 18-h fasted *db/db* male mice and primary hepatocytes using TRIzol reagent according to manufacturer

instructions (Sigma-Aldrich). The quantitative PCR (qPCR) primer sequences used are listed in the Table 1.

Western blotting

Antibodies against MT-CO1 (Abcam), UQCRC1, FOXO1, NRF1 and TFAM (Cell Signaling Technology) were used according to the manufacturer's instructions. Protein densitometry was performed and analyzed using ImageJ.

Mouse primary hepatocyte isolation and cell culture

Primary mouse hepatocytes were isolated from 8–12 weeks old, random-fed mice and cultured in DMEM with 10% FBS as previously described (Zhang *et al.* 2012). HepG2 cells were cultured with DMEM supplemented with 10% FBS and 1% antibiotics in 37°C incubator with 5% CO₂. HepG2 cells transfection was performed by lipofectamine 3000 reagent according to the manufacturer's instructions (Thermo Fisher).

Quantification of mtDNA copy number by qPCR

Total DNA was extracted from primary hepatocytes treated with 100 nM glucagon for 10 h by DNeasy Blood and tissue kit (QIAGEN). qPCRs were carried out by using COX3 and β 2 microglobulin (β 2m) primers as described previously (Khiati *et al.* 2015).

Mitochondrial stress test in primary hepatocytes and HepG2 cells

Primary hepatocytes (8000 cells/well) and HepG2 cells (20,000 cells/well) were seeded into the XF96 microplates. After treating with 100 nM glucagon, culturing DMEM medium was changed with XF base medium supplied with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose. Then microplate was placed in non-CO₂ 37°C incubator for 1 h before loading to the XF96 Extracellular Flux Analyzer (Seahorse Bioscience). Oxygen consumption rate was measured in Analyzer. All measurements were performed following manufacturer's instructions and protocols. The results were normalized by the total proteins each well.

Fatty acid oxidation analysis

Isolated primary hepatocytes were seeded into the XF96 cell culture microplate, as mentioned above. 24 h prior to the assay, replace growth medium with substrate-limited medium (DMEM with 0.5 mM glucose, 1 mM

Table 1 Primers for quantitative PCR (qPCR).

Gene name	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
Mouse		
<i>Foxo1</i>	AGGAACTGAGGAGCAGTCCA	ATGATTCCAATGGCACAGT
<i>Fxn</i>	TTGAAGACCTCGCAGACAAGC	AGGCCAATGAAGACAAGTCCA
<i>Urod</i>	TTTGCACTGCCCTACATTCTGT	AGCCGACCGATCTCTTCTCA
<i>Mt-nd6</i>	CACAACTATATATTGCCGCTACC	GCTACTGAGGAATATCCAGAGAC
<i>Sdha</i>	TGCTGGAGAAGAATCGGTTATG	ACAGCATCAGATTCTGCAGCTCC
<i>Uqcrc1</i>	ACCACTGCTACAGAGAGTGAGG	AGCATCTGGGCATCCACCTCTG
<i>Mt-co1</i>	GTCTGATCCGTACTTATTACAG	GCTCATACTATTCTATATAGCCG
<i>Atp5a</i>	GAGACTGGGCGTGTGTTAAGCA	CATTACCGAGGGCGTCAACCAC
<i>Nrf1</i>	CAGACGACGCAAGCATCAGAG	GCTCCGACGGCTGCTGCGGTTTC
<i>Tfam</i>	GCACTGGTAAAGAGAAGAGAAT	TCCTATCATCTTTAGCAAGCTG
<i>Vlca</i>	CCTTGGTGTAGCGTTACCT	GGCTACATCGGATCCACTCG
<i>Cpt1a</i>	GCCGATCATGGTTAACGCAACT	AGACCTTGAAGTAACGGCTC
<i>Hadha</i>	CGTATGCCATGACTGTCCCA	CCAAATTTCTGCGATTACAGCAAG
<i>Acadm</i>	TTCATTGTGGAAGCCGACACC	TCCTATCCAGGGCATACTTCG
<i>Fis1</i>	CCGGCTCAAGGAATATGAAA	CCATGCCTACCAGTCCATCT
<i>Drp1</i>	CGGTTCCCTAAACTTCACGA	GCACCATTTTCATTGTCACG
<i>Mfn1</i>	TCTCCAGCCCAACATCTTCA	ACTCCGGCTCCGAAGCA
<i>Mfn2</i>	GGGGCTACATCCAAGAGAG	GCAGAACTTTGTCCAGAGC
<i>Sirt1</i>	ACTTGCCATCATGAAGCCAGA	TATTTGAGGCACTTATGCGGTA
<i>Ppargc1a</i>	CTACCGTTACACCTGTGACG	AGTTGGTATCTGAATCTGCA
<i>18s</i>	AGCCTGCGGCTTAATTTGAC	CAACTAAGAACGGCCATGCA
Human		
<i>FXN</i>	CCAAACAAGCAAATCTGGCTA	GGGCATCAAGCATCTTTTCCG
<i>UROD</i>	TTTGCACTGCCTTACATCCGT	AACTGCCCGATCTCCTCTCA

GlutaMAX, 0.5 mM carnitine and 1% FBS). 45 min before the assay, wash cells and replace with FAO assay medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 2 mM MgSO₄, 1.2 mM NaH₂PO₄, 2.5 mM glucose, 0.5 mM carnitine and 5 mM HEPES) and incubate in a non-CO₂ incubator for 30–45 min at 37°C. Prior to the assay, add 30 µL XF Palmitate-BSA FAO substrate or BSA to the appropriate wells. Then immediately insert the XF cell culture microplate into the XF96 Analyzer and run the XF Cell Mito Stress Test. The results were normalized by the total proteins each well.

Heme measurement

25 mg liver tissues were collected from 18 h fasted control and *db/db* mice and then homogenized in 400 µL 1% TBST. The heme level was measured by Heme Assay Kit (Sigma-Aldrich).

Affymetrix GeneChip analysis

Gene expression was determined on MG4302.0 GeneChip (Affymetrix). Livers in control and L-F1KO mice fasted for 18 h were collected (*n*=2/group). RNA was extracted by using TRIzol (Invitrogen). 15 µg of each RNA sample was used for labeling and hybridization which was performed

at the Harvard Medical School Children's Hospital Boston Genetic Core Facility. Affymetrix microarray Suite 5.0 was used to generate cell intensity files that were analyzed by Transcriptome Analysis Console 4.0 (Zhang *et al.* 2012).

ATP measurement

Primary mouse hepatocytes were seeded into 96-well plate in DMEM medium with 10% FBS and 1% penicillin/streptomycin at 10,000 cells/well. After fasting 3 h, primary mouse hepatocytes were treated with 100 nM glucagon for 10 h. The amount of ATP was measured by ATPlite Luminescence ATP Detection Assay System (PerkinElmer) according to the instruction.

Hepatic metabolite analysis

The liver samples from 8- to 12-week-old random-fed control and L-F1KO mice intraperitoneally injected with or without 16 µg/kg glucagon for 1 h were collected (*n*=8). The global metabolic profiles in livers from different treatments were examined by Metabolon Inc. (Durham, NC, USA). Samples were prepared using the automated MicroLab STAR system from Hamilton Company. Samples were measured by ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and gas chromatography-mass spectroscopy (GC-MS).

Electron microscopy

Random-fed control and L-F1KO mice were injected with 16 µg/kg glucagon for 1 h. The livers collected were perfused with 2% paraformaldehyde solution containing 2.5% glutaraldehyde. The perfused livers were cut into small pieces (1 × 1 × 2 mm) and were fixed in 5% glutaraldehyde at 4°C overnight. Then, they were fixed in 1% osmium tetroxide for 1 h at 25°C, stained in 2% uranyl acetate and dehydrated in a series of ethanol dilution (50–100%), finally embedded in epoxy resin. The sections (70 µm) were stained with 2% uranyl acetate and lead citrate.

Statistical analysis

Results are presented as the mean ± S.E.M. Data for two groups were analyzed by two-tailed Student's *t* test to determine *P* values. ANOVA was used to determine *P* values for data with three or more groups. *P* < 0.05 was considered statistically significant.

Results

FOXO1 is required for the effect of glucagon on hepatic mitochondrial function

We firstly assessed the role of glucagon in hepatic mitochondrial oxidative phosphorylation in hepatocytes. HepG2 cells were pretreated with 100 nM glucagon for 2, 6 and 10-h, respectively. Subsequently, oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and antimycin A (AA) were added, then oxygen consumption rate measured by the Seahorse XFe96 Analyzer. At the basal level, oxygen consumption rate was reduced by 15, 25 and 30%, respectively, in HepG2 cells when treated with glucagon for 2, 6 and 10-h (*P* < 0.01). Moreover, compared with the vehicle group, 2, 6 and 10-h treatments of glucagon resulted in 18, 27 and 35% decrease in FCCP-induced maximum oxygen consumption rate in HepG2 cells, respectively (*P* < 0.05; Fig. 1A).

Glucagon suppressed the oxygen consumption rate in HepG2 cells in a time-dependent manner. Therefore, we treated mouse primary hepatocytes with glucagon for 10 h to assess the effect of glucagon on hepatic mitochondrial function. ATP production was measured in primary hepatocytes and the result showed that glucagon reduced ATP production by 35% in the control hepatocytes (*P* < 0.05). However, the suppression of ATP level by glucagon treatment was abolished in the L-F1KO hepatocytes (Fig. 1B). Oxygen consumption rate was

further analyzed in primary hepatocytes. The control hepatocytes treated with glucagon exhibited significant reduction in oxygen consumption rate compared with that of vehicle group at different time points (*P* < 0.05; Fig. 1C). Further analysis showed that glucagon decreased the basal respiration, maximal respiration level, ATP production and spare respiration by 34, 39, 44 and 45%, respectively, in control hepatocytes (*P* < 0.05, Fig. 1D).

However, L-F1KO hepatocytes treated with and without glucagon exhibited comparable oxygen consumption rate at different time points (Fig. 1E). Likewise, L-F1KO hepatocytes showed no significant differences between the vehicle and glucagon treatment group in the basal respiration, the maximal respiration, ATP production and the spare respiration levels (Fig. 1F). In the primary hepatocytes of diabetic model *db/db* mice, oxygen consumption rate was decreased significantly in both basal level and FCCP-induced level (*P* < 0.05; Fig. 1G). Moreover, compared with control hepatocytes, *db/db* hepatocytes showed 42, 44, 54 and 57% decrease in the basal respiration, maximal respiration level, ATP production and spare respiration, respectively (*P* < 0.05; Fig. 1H). Taken together, the data suggest that FOXO1 mediates the negative role of glucagon in hepatic mitochondrial oxygen consumption and oxidative phosphorylation.

FOXO1 regulates expression of heme biosynthesis genes

We then detected the effect of glucagon on the expression of FOXO1 in mouse primary hepatocytes. The results showed that glucagon significantly increased the expression of FOXO1 in both mRNA and protein level (*P* < 0.05; Fig. 2A and B). To explore the downstream genes responsible for mitochondrial dysfunction, the transcription profiles in the liver of control and L-F1KO mice were analyzed by the GeneChip microarray. About 2129 genes were differentially expressed between the livers of control and L-F1KO mice (Fig. 2C). Pathway analysis showed that differential expression genes were located at insulin signal pathways, which is consistent with the function of FOXO1 in the glucose metabolism. Further function analysis revealed that the differential expression genes were clustered to acetylation, transcription regulation, mitochondrial and heme biosynthesis (Fig. 2D). Fifty-four differential expression genes were associated with mitochondrial functions, which include the heme biosynthesis genes, Frataxin (*Fxn*) and uroporphyrinogen decarboxylase (*Urod*). Then we verified the expression of

Figure 1

Glucagon decreases mitochondrial function and oxygen consumption via FOXO1. (A) Oxygen consumption rate (OCR) was measured in HepG2 cells treated with 100 nM glucagon. HepG2 cells were cultured in DMEM medium supplied with 10% FBS and 1% P/S. Cells were treated with 100 nM glucagon for 2, 6, and 10 h. OCR was then measured. $^{*}P < 0.01$ vs vehicle; $^{*}P < 0.05$, $^{##}P < 0.01$ vs vehicle; $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle, $n = 4$. (B) ATP production was measured in control (CNTR) and L-F1KO primary mouse hepatocytes cultured in DMEM medium with 10% FBS and 1% P/S. Cells were treated with 100 nM glucagon for 10 h and ATP production was measured by the ATP lite Luminescence, $^{*}P < 0.05$ vs CNTR-Vehicle; $^{*}P < 0.05$ vs CNTR-Glucagon, $n = 5$. (C) OCR was measured in the control primary mouse hepatocytes treated with or without 100 nM Glucagon for 10 h and OCR measured by seahorse analyzer. $^{*}P < 0.05$, $^{**}P < 0.01$ vs CNTR-Glucagon. (D) Basal respiration, maximal respiration, ATP production, and respiration capacity were calculated by OCR in control hepatocytes. $^{*}P < 0.05$, $^{**}P < 0.01$ vs CNTR-Vehicle, $n = 3-5$. (E) OCR was measured in L-F1KO hepatocytes with or without glucagon. L-F1KO hepatocytes were cultured in DMEM medium supplied with 10% FBS and 1% P/S, then treated with or without 100 nM glucagon for 10 h and OCR measured. (F) Basal respiration, maximal respiration, ATP production, and respiration capacity were calculated by OCR in L-F1KO hepatocytes. (G) OCR was measured in the control and *db/db* primary mouse hepatocytes. Hepatocytes were cultured in DMEM medium supplied with 10% FBS and 1% P/S, then OCR measured by seahorse analyzer. $^{*}P < 0.05$, $^{**}P < 0.01$ vs CNTR, $n = 4-7$. (H) Basal respiration, maximal respiration, ATP production, and respiration capacity were calculated by OCR in control and *db/db* hepatocyte. $^{*}P < 0.05$, $^{**}P < 0.01$ vs CNTR, $n = 4-7$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0081>.

Fxn and *Urod* responsible for heme biosynthesis in the liver of control and L-F1KO mice by qPCR. The expression of *Foxo1* was reduced by 80% in the liver of fasted L-F1KO mice compared with that in control mice ($P < 0.01$). The L-F1KO livers exhibited 1.6-fold increase in *Fxn* and two-fold upregulation in *Urod* compared with the control counterpart ($P < 0.01$; Fig. 2E). Thus, FOXO1 plays a role in suppression of heme biosynthetic genes *Fxn* and *Urod*.

Glucagon reduces heme generation through FOXO1

We then asked whether glucagon decreases expression of genes responsible for heme biosynthesis and then inhibits heme generation. Mouse primary hepatocytes

were isolated from the control and L-F1KO mice and then treated with 100nM glucagon for 10h. In control hepatocytes, glucagon decreased *Fxn* and *Urod* mRNA expression by 32 and 22%, respectively, compared with the vehicle group ($P < 0.05$). However, glucagon had no significant effect on the expression levels of *Fxn* and *Urod* in L-F1KO hepatocytes (Fig. 3A). Overexpression of FOXO1 in HepG2 cells resulted in 26 and 20% decrease in *FXN* and *UROD* expression, compared with control group ($P < 0.05$). Glucagon treatment for 10h in HepG2 cells also decreased the expression of *FXN* and *UROD* by 23 and 21%, respectively ($P < 0.05$). However, there were no significant differences in *FXN* and *UROD* expression between FOXO1 overexpression and glucagon treatment

Figure 2

Heme biosynthesis genes, *Fxn* and *Urod*, are regulated by FOXO1 in the liver. (A) Relative mRNA level of *Foxo1* was determined by qPCR in control primary mouse hepatocytes treated with or without 100 nM glucagon. * $P < 0.05$, ** $P < 0.01$ vs 0 min treatment, $n = 3$. (B) FOXO1 protein level was measured by Western blot in primary mouse primary hepatocytes. * $P < 0.05$, ** $P < 0.01$ vs 0 min treatment, $n = 3$. (C) Scatter plot of differentially expressed genes between livers from 18 h-fasted control and L-F1KO male mice. (D) Functional analysis of differentially expressed genes in the liver of 18 h-fasted control and L-F1KO male mice. (E) Relative mRNA levels of *Foxo1*, *Fxn*, and *Urod* between livers of 18 h-fasted control and L-F1KO male mice. ** $P < 0.01$ vs CNTR, $n = 3$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0081>.

groups (Fig. 3B). We then analyzed the metabolites in the liver of random-fed control and L-F1KO mice intraperitoneally (i.p.) injected with glucagon. The principal component analysis indicated that there were distinct separations among different groups (Fig. 3C). Glucagon treatment in control mice decreased heme level by 50% in the liver ($P < 0.05$). However, the heme level in L-F1KO livers was barely affected by glucagon administration and was comparable to the heme level in control mice without glucagon injection (Fig. 3D). Further analysis in *db/db* mice showed that compared with control mice, the expression levels of *Fxn* and *Urod* were decreased by 31 and 28%, respectively in the liver of *db/db* mice ($P < 0.01$; Fig. 3E). Heme level in *db/db* mice liver was

decreased by 39% ($P < 0.05$; Fig. 3F). Thus, these results demonstrate that glucagon downregulates the expression of *Fxn* and *Urod* and inhibits heme biosynthesis in a FOXO1-dependent manner

Glucagon impairs integrity of mitochondrial electronic transport chain via FOXO1

Heme is an important molecule that can affect the function of complexes III and IV to maintain mitochondrial function (Atamna *et al.* 2001, 2007, Cheng *et al.* 2009). We hypothesized that glucagon might affect the integrity of mitochondrial electron transport chain (ETC) through suppressing heme production. The expression of

Figure 3

Glucagon reduces hepatic heme pool by decreasing gene expression of *Fxn* and *Urod*. (A) Relative mRNA levels of *Fxn* and *Urod* in control and L-F1KO primary mouse hepatocytes treated with or without 100 nM glucagon for 10 h. * $P < 0.05$ vs CNTR-Vehicle; # $P < 0.05$ vs CNTR-Glucagon, $n = 3$. (B) Relative mRNA expression of *FXN* and *UROD* was detected in HepG2 cells transfected with 4 μ g plasmid DNA expressing control green fluorescence protein (GFP) or FOXO1 and treated with 100 nM glucagon for 10 h. *FXN* and *UROD* mRNA levels were measured by qPCR. * $P < 0.05$ vs group without Glucagon treatment and FOXO1 overexpression. (C) Principal component analysis of metabolites in the liver from random-fed control and L-F1KO male mice i.p. injected with 16 μ g/kg glucagon for 1 h. (D) The relative value of Heme in the livers of random-fed control and L-F1KO male mice i.p. injected with 16 μ g/kg glucagon for 1 h. * $P < 0.05$ vs CNTR-Vehicle; # $P < 0.05$ vs CNTR-Glucagon, $n = 5$. (E) Relative mRNA levels of *Fxn* and *Urod* in the fasted control and *db/db* mice livers. ** $P < 0.01$ vs CNTR, $n = 3-4$. (F) The relative value of Heme in the fasted livers of control and *db/db* mice livers. * $P < 0.05$ vs CNTR, $n = 3-4$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0081>.

complexes I/II/III/IV/V in ETC, relatively indicated by NADH dehydrogenase subunit 6 (MT-ND6), succinate dehydrogenase complex, subunit A (SDHA), ubiquinol-cytochrome c reductase core protein (UQCRC1), cytochrome c oxidase subunit 1 (MT-CO1) and alpha subunit 1 (ATP5A), were examined in control and L-F1KO hepatocytes treated with glucagon. Glucagon decreased the mRNA expression of *Mt-co1* and *Uqcrc1* by 67 and 50%, respectively, in control hepatocytes ($P < 0.05$) and had no effects on *Mt-nd6*, *Sdha* and *Atp5a* in both control and L-F1KO hepatocytes. However, the effect of glucagon on the suppression of *Uqcrc1* expression was blocked and the inhibition of *Mt-co1* expression by glucagon largely attenuated by 35% in L-F1KO hepatocytes ($P < 0.05$,

Fig. 4A). We further analyzed the protein abundance of total FOXO1, MT-CO1 and UQCRC1 in the control and L-F1KO hepatocytes by Western blotting. Glucagon treatment increased protein level of total FOXO1 by 30% in control hepatocytes ($P < 0.05$). Glucagon reduced UQCRC1 protein level by 36% in the glucagon-treated control hepatocytes ($P < 0.05$), which was abolished in the L-F1KO hepatocytes. Compared with the vehicle group, MT-CO1 protein abundance was reduced by 64% in the glucagon-treated control hepatocytes ($P < 0.05$), and the reduction of MT-CO1 protein level by glucagon was attenuated by 45% in L-F1KO hepatocytes ($P < 0.05$; Fig. 4B and C). We also observed 36 and 40% decrease in UQCRC1 and MT-CO1, respectively, in *db/db* mice

Figure 4

Glucagon decreases hepatic mitochondrial ETC integrity. (A) The relative mRNA expression of *Mt-nd6* (complex I), *Sdha* (complex II), *Uqcrc1* (complex III), *Mt-co1* (complex IV), *Atp5a* (complex V) in control and L-F1KO primary hepatocytes treated with or without 100 nM glucagon for 10 h. * $P < 0.05$ vs CNTR-Vehicle; # $P < 0.05$ vs CNTR-Glucagon. (B and C) The protein levels of FOXO1, UQCRC1 and MT-CO1 in control and L-F1KO primary hepatocytes with or without 100 nM glucagon for 10 h were determined by Western blotting and (C) quantification of FOXO1, UQCRC1 and MT-CO1 protein levels. * $P < 0.05$, ** $P < 0.01$ vs CNTR-Vehicle; # $P < 0.05$, ## $P < 0.01$ vs CNTR-Glucagon. (D and E) The protein levels of UQCRC1 and MT-CO1 in the fasted control and *db/db* mice livers were determined by Western blotting and (E) quantification of UQCRC1 and MT-CO1 protein levels. * $P < 0.05$, ** $P < 0.01$ vs CNTR.

liver ($P < 0.05$; Fig. 4D and E). These results demonstrate that glucagon disrupts complex III and complex IV and impairs the integrity of mitochondrial ETC partially through FOXO1.

Glucagon promotes fatty acid oxidation to compensate for hepatic energy deficit

Glucagon level goes up to promote hepatic glucose production during fasting state. To identify whether glucagon plays a similar role in hepatic mitochondria during fasting state, we detected the expressions of *Fxn*, *Urod*, *Uqcrc1* and *Mt-co1* in the livers of 18-h fasted and random-fed mice. The fasted L-F1KO liver exhibited 30% higher in *Fxn* and 35% higher expression in *Urod* than that of fasted control liver ($P < 0.05$). The expression of *Fxn* and *Urod* in the fed control liver was increased by 28 and 26%, respectively, compared with fasted control liver ($P < 0.05$). However, there were no significant differences in the expression of *Fxn* and *Urod* between control and L-F1KO liver under the feeding condition (Fig. 5A and B). Compared with the fed liver of control mice, UQCRC1 protein level was decreased by 44% in the fasted liver of control mice ($P < 0.05$). In the fasted condition, FOXO1

deficiency resulted in a 47% increase in the UQCRC1 expression in the liver ($P < 0.05$), which is not observed in fed state. However, there were no significant changes in MT-CO1 protein level among different groups (Fig. 5C and D). Thus, glucagon tends to temporally and slightly attenuate the hepatic mitochondrial function during fasting state.

Glucagon-induced hepatic glucose production requires energy supply. The attenuated hepatic mitochondrial activity might be a paradox for glucagon-triggered hepatic glucose production in the liver during fasting state. To solve this inconsistency, we hypothesize that glucagon promotes fatty acid oxidation, which compromises the decreased mitochondrial function temporally to ensure hepatic glucose production. To prove this hypothesis, we detected the oxygen consumption level in primary hepatocytes cultured in the medium with palmitate. The addition of palmitate increased the oxygen consumption rate significantly in both control hepatocytes with and without glucagon treatment, compared with group without palmitate ($P < 0.01$). Importantly, glucagon barely reduced oxygen consumption rate in palmitate-added control hepatocytes. However, glucagon decreased oxygen consumption rate in control group without

Figure 5

The expression of FXN, UROD, UQCRC1, and MT-CO1 in the mice livers under fasting and feeding state. (A and B) Relative mRNA levels of *Fxn* and *Urod* in liver from 18 h-fasted and random-fed control and L-F1KO male mice. * $P < 0.05$ vs CNTR-Vehicle. (C and D) The protein levels of MT-CO1 and UQCRC1 in the livers of random-fed and 18 h-fasted control and L-F1KO male mice were detected via Western blotting and (D) quantification of UQCRC1 and MT-CO1 protein levels. * $P < 0.05$ vs Fasted-CNTR.

palmitate ($P < 0.05$, Fig. 6A). Further analysis showed that addition of palmitate in control hepatocyte without glucagon treatment increased basal respiration and ATP production by 77 and 67%, respectively ($P < 0.01$). When pretreated with glucagon, the control hepatocytes with addition of palmitate showed 144 and 125% increase in basal respiration and ATP production, respectively, compared with that in groups without palmitate addition ($P < 0.01$). In control hepatocytes without palmitate, glucagon decreased basal respiration and ATP production by 28 and 26%, respectively ($P < 0.05$). However, in the presence of palmitate treatment, glucagon failed to decrease the basal respiration and ATP production (Fig. 6B). Similarly, L-F1KO hepatocytes with palmitate showed significantly increased oxygen consumption rate ($P < 0.01$). In particular, glucagon treatment increased the FCCP-induced oxygen consumption rate in the presence of palmitate ($P < 0.05$; Fig. 6C). Basal respiration and ATP production were elevated by 46 and 105%, respectively, in L-F1KO hepatocytes with the addition of palmitate but without glucagon treatment ($P < 0.01$). After treatment with glucagon, L-F1KO hepatocytes added with palmitate exhibited 85 and 185% increase in basal respiration and ATP production, respectively ($P < 0.01$). Glucagon treatment lead to 21% increase in ATP production of the L-F1KO hepatocytes with palmitate ($P < 0.05$, Fig. 6D). The fatty acid oxidation genes *Hadha*, *Cpt1a*, *Vlcad* and *Acadm* were also upregulated by 26, 360, 80 and 32%, respectively in 10-h glucagon-treated control hepatocytes ($P < 0.05$). Compared with control hepatocytes, L-F1KO hepatocytes showed 56, 56, 70 and 34% increase in *Hadha*, *Cpt1a*, *Vlcad* and *Acadm* mRNA expression, respectively

($P < 0.05$). L-F1KO hepatocytes showed 3.6- and 1.9-fold increases by glucagon in *Cpt1a* and *Vlcad* ($P < 0.01$, Fig. 6E). Fasted control and L-F1KO mice exhibited 2–4 folds higher level of fatty acid oxidation genes, *Hadha*, *Cpt1a*, *Vlcad* and *Acadm*, than that in fed mice livers ($P < 0.01$). The FOXO1 deficiency in liver induced a moderate but not significant increase in the expression of *Hadha*, *Cpt1a* or *Vlcad* during the fasting state (Fig. 6F). Taken together, glucagon-suppressed oxygen consumption or mitochondrial function is secondary to the substrate-driven fatty acid oxidation in the liver, which can dominate during fasting state when fatty acid influx to the liver increases.

Glucagon attenuates hepatic mitochondrial biogenesis

We then asked whether glucagon regulates mitochondrial biogenesis. We detected the mitochondrial copy numbers in the hepatocytes using qPCR. Glucagon greatly decreased the mitochondrial copy number by 29% in control hepatocytes ($P < 0.05$), but the effect of glucagon on L-F1KO hepatocytes was diminished (Fig. 7A). The electron microscopy of liver sections from random-fed control mice injected with 16 $\mu\text{g/kg}$ glucagon revealed larger and fewer mitochondria than liver sections from untreated control mice. However, both L-F1KO mice injected with and without glucagon showed comparable hepatic mitochondria size and number, which is similar with that in control mice not injected with glucagon (Fig. 7B). We further examined the expression levels of nuclear respiratory factor-1 (*Nrf1*) and mitochondrial

Figure 6

Glucagon promotes fatty acid oxidation to compensate energy deficit. (A, B, C and D) Fatty acid oxidation (FAO) was measured in primary hepatocytes. The hepatocytes were cultured in substrate-limited medium 24 h prior to the assay and treated with 100 nM glucagon for 10 h. FAO assay medium was used to culture hepatocytes 45 min before the assay and then palmitate was added into wells just before assay, followed by OCR measurement. Fatty acid oxidation in control primary hepatocytes (A). * $P < 0.05$; ** $P < 0.01$ vs CNTR-Vehicle; ## $P < 0.01$ vs CNTR-Glucagon. Fatty acid oxidation in L-F1KO primary hepatocytes (C). ** $P < 0.01$ vs L-F1KO-Glucagon; ## $P < 0.01$ vs L-F1KO-Vehicle; § $P < 0.05$ vs L-F1KO-Vehicle-Palmitate. Basal respiration and ATP production were calculated by OCR in control (B) and L-F1KO (D) hepatocytes. * $P < 0.05$, ** $P < 0.01$ vs indicated; # $P < 0.05$ vs L-F1KO-Vehicle-Palmitate. (E) The mRNA expressions of *Hadha*, *Cpt1a*, *Vlcad*, and *Acadm* were measured in control and L-F1KO hepatocytes treated with or without 100 nM glucagon for 10 h. * $P < 0.05$, ** $P < 0.01$ vs CNTR-Vehicle; ## $P < 0.01$ vs L-F1KO Vehicle. (F) The mRNA expressions of *Hadha*, *Cpt1a*, *Vlcad*, and *Acadm* in livers of random-fed and 18 h-fasted control and L-F1KO male mice were measured by qPCR. * $P < 0.05$, ** $P < 0.01$ vs indicated. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0081>.

transcriptional factor A (*Tfam*), two key genes for mitochondrial function and biogenesis. After 10-h treatment, glucagon suppressed the mRNA expression of *Nrf1* and *Tfam* by 21 and 33%, respectively, in the control hepatocytes, rather than in the L-F1KO hepatocytes ($P < 0.05$, Fig. 7C). At the protein levels, glucagon treatment similarly increased FOXO1 protein by 40% in control hepatocytes ($P < 0.05$). Glucagon decreased NRF1 and TFAM protein level by 49 and 44%, respectively, in control hepatocytes, while loss of FOXO1 in L-F1KO hepatocytes abolished the suppressing effect of glucagon on NRF1 and TFAM expression ($P < 0.05$, Fig. 7E and F).

Then, we detected the expression of NRF1 and TFAM in the *db/db* mice livers. Compared with the liver of control mice, mRNA expression levels of *Nrf1* and *Tfam* were decreased by 40 and 36% in the *db/db*

mice liver ($P < 0.05$; Fig. 7D). Similarly, *db/db* mice liver showed 30 and 40% decrease in NRF1 and TFAM protein, respectively, compared with that in control mice ($P < 0.01$; Fig. 7G and H). PPARGC1A plays an important role in mitochondrial biogenesis. We found that glucagon increased the expression of *Ppargc1a* by 5- and 2.5-fold in control and L-F1KO primary hepatocytes, respectively ($P < 0.01$; Fig. 7I). Considering the activity of PPARGC1A is induced by SIRT1-mediated deacetylation, we examined the *Sirt1* level in primary hepatocytes. The result showed that glucagon treatment decreased *Sirt1* expression by 33% in control hepatocytes but not in L-F1KO hepatocytes ($P < 0.05$; Fig. 7I). We also detected the hepatic mitochondrial fission and fusion genes. Glucagon increased the expression of Mitofusin 2 (*Mfn2*) by 2.2-fold in control hepatocytes rather than in L-F1KO hepatocytes

($P < 0.05$). However, we did not observe the effect of glucagon on the levels of Fission 1 (*Fis1*), Dynamin-related protein 1 (*Drp1*) and *Mfn1* in hepatocytes (Fig. 7J). Together, these results suggest that glucagon potentially attenuates hepatic mitochondrial biogenesis and dynamics involved in control of NRF1, TFAM and MFN2 expression, which is dependent on FOXO1.

Discussion

Glucagon is traditionally an anti-hypoglycemic hormone, cooperating with insulin to govern the metabolic homeostasis. Glucagon excess is essential for the development of diabetes. High level glucagon contributes

to hyperglycemia in diabetic animals and humans. In this study, we demonstrated that glucagon, especially in long-term hyperglucagonemia condition, decreases the expression of genes responsible for mitochondrial biogenesis and function in a FOXO1-dependent manner.

FOXO1, a key mediator of insulin signaling in control of blood glucose homeostasis, also regulates mitochondrial function in the liver. Deletion of FOXO1 can rescue the dysfunctional mitochondria in the liver deficient for IRS1 and IRS2 in mice (Cheng *et al.* 2009). We demonstrated that glucagon increases FOXO1 activity by promoting its nuclear translocation and stability via phosphorylation of FOXO1 at Ser 273 (Wu *et al.* 2018). We also observed that glucagon increased FOXO1 expression in both mRNA and protein level. Our results in this study further showed that

FOXO1 is an important mediator in glucagon signaling in control of mitochondrial biogenesis and function.

Heme is a cofactor used commonly for multiple biological reactions (Kim *et al.* 2012). Heme and iron-sulfur clusters are required by proteins in the mitochondrial ETC for correct folding and assembly into a functional complex with full activity (Atamna *et al.* 2002). In our study, glucagon administration reduced heme production depending on FOXO1. The reduced level of heme is possibly responsible for the downregulated hepatic oxygen consumption by glucagon. Glucagon suppressed the expression of FXN and UROD via FOXO1 to regulate heme pool. Frataxin is a conserved mitochondrial protein and involved in cellular iron metabolism. Frataxin acts to protect bioavailable iron within mitochondria and to facilitate its use for heme synthesis (Zhang *et al.* 2005). Frataxin is also associated with mitochondrial membranes and involved in mitochondrial iron homeostasis. Overexpression of frataxin in mammalian cells increased mitochondrial membrane potential and resulted in the elevation of cellular ATP content (Ristow *et al.* 2000). UROD participates in heme biosynthetic process by catalyzing conversion of URO'GEN to coproporphyrinogen III (Ajioka *et al.* 2006). Dysregulation of UROD expression results in retardation of heme maturation. Therefore, the downregulated expression of FXN and UROD by glucagon can contribute to a reduction of heme biosynthesis in the liver and affect hepatic mitochondrial function.

Oxidative phosphorylation is driven by proteins located within the mitochondrial inner membranes and ETC (Wallace 1999). Heme plays an important role in mitochondrial oxidation by promoting electron transport and maintaining expression and stability of complexes III and IV (Atamna *et al.* 2001, Cheng *et al.* 2009). We observed that only UQCRC1 and MT-CO1 (heme-dependent) were impaired after glucagon treatment, indicating the decrease in mitochondrial heme pool by glucagon. Therefore, glucagon can intervene the integrity of ETC by regulating UQCRC1 and MT-CO1 to decrease the mitochondrial oxidative phosphorylation.

During the fasting condition, insulin level falls and glucagon becomes dominant for increasing gluconeogenesis and glycogenolysis, which requires energy supply. Attenuated hepatic mitochondrial activity by glucagon can lead to temporal energy deficit, which might impair the ability of glucagon to promote glucose production in the liver. However, during the fasting status, glucagon stimulates the liver to utilize free fatty acid efficiently providing energy for glucose production

and conversion of fatty acid into ketone bodies (von Meyenn *et al.* 2013). Fatty acid oxidation assay in mouse hepatocytes showed that palmitate, largely triggered fatty acid β -oxidation to maintain normal energy production, overriding the glucagon-suppressed mitochondrial function. Therefore, although increased secretion of glucagon under the fasting condition, the increase of fatty acid flux from lipolysis of adipose tissue majorly governs the metabolic regulation and mitochondrial function with enhanced fatty acid oxidation, so that temporal glucagon-suppressed mitochondrial function is secondary to the metabolic demand as adaptive compensation.

Patients with type 2 diabetes typically show normal-to-high fasting glucagon level that fails to properly decrease after nutrition intake (Müller *et al.* 2017). Continuously high level of glucagon was supposed to play roles in the development of chronic hyperglycemia and diabetes. Inhibition of glucagon action can alleviate hyperglycemia in type 2 diabetes (Liang *et al.* 2004). Hepatic mitochondrial function and biogenesis were inhibited in *db/db* mice, which can be recovered by the deletion of FOXO1 (Cheng *et al.* 2009). Our previous research showed that *db/db* mice exhibited 2–3 times higher in blood glucagon level than that in wild-type mice (Wu *et al.* 2018). Our study shows that *db/db* mice displayed significantly decreased hepatic mitochondrial function, which is possibly caused by hyperglucagonemia. Therefore, long-term high glucagon level might cause severe defect of hepatic mitochondrial function, resulting in energy deficit that cannot be compensated by substrate-increased fatty acid oxidation. More seriously, dysfunctional hepatic mitochondria can impair fatty acid oxidation activity, resulting in fatty liver diseases. Thus, managing glucagon level into a reasonable range is a critical therapy for the management of type 2 diabetes, not only because of controlling blood glucose but also rescuing hepatic mitochondrial function.

PPARGC1A plays a role in mitochondrial function and biogenesis by triggering the expression of nuclear respiration factor-1 (NRF1) and mitochondrial transcriptional factor A (TFAM) (Liu *et al.* 2011). Glucagon treatment increased the expression of *Ppargc1a*. However, NRF1 and TFAM were downregulated after glucagon treatment. This paradox might be explained by the acetylation of PPARGC1A. PPARGC1A is heavily acetylated by acetyltransferase GCN5, inhibiting its activity. SIRT1, modulated by NAD⁺/NADH, deacetylates PGC1 α to increase its activity on transcriptional targets (Scarpulla 2011). We observed that glucagon decreased *Sirt1* mRNA level in a FOXO1-dependent manner.

Figure 8

A schematic diagram representing the function of glucagon in diabetic state. In diabetic condition, glucagon maintains at high level to significantly increase hepatic glucose production by glycogenolysis and gluconeogenesis. Meanwhile, high level of glucagon decreases the expression of FXN and UROD in a FOXO1-dependent manner to reduce the heme pool, impairing heme-dependent UQCRC1 (complex III) and MT-CO1 (complex IV). Moreover, glucagon also decreases NRF1 and TFAM to attenuate the hepatic mitochondrial biogenesis in a FOXO1-dependent manner. Therefore, hyperglucagonemia suppresses the hepatic mitochondrial function and biogenesis. The hyperglycemia and dysfunctional hepatic mitochondria rendered by hyperglucagonemia will further lead to other liver diseases. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0081>.

Therefore, PPARGC1A might maintain high level of acetylation and its activity remains suppressed. However, we cannot exclude the possibility that FOXO1 plays a role in regulating expression NRF1 and TFAM directly. Mitochondrial constantly adjust their shapes by fusion, fission, and motility in response to the cellular energy status (Youle *et al.* 2012). Fusion process allows one mitochondrion to compensate for other mitochondrial dysfunction by sharing transfer RNAs, ribosomal RNAs or proteins (Nasrallah *et al.* 2014). Increased fusion gene (*Mfn2*) by glucagon potentially stimulated hepatic mitochondrial fusion to rescue their function defects caused by glucagon. Therefore, during the fasting period, glucagon tends to regulate hepatic mitochondrial dynamics and biogenesis, which will be recovered in the feeding state. However, long-term high glucagon level,

especially in type 2 diabetic patients, possibly will cause significant defects in hepatic mitochondrial biogenesis.

Glucagon primarily maintains blood glucose by inducing gluconeogenesis and glycogenolysis, and promotes fatty acid oxidation to ensure energy supply during the fasting condition (Habegger *et al.* 2010, von Meyenn *et al.* 2013). In diabetic status, long-term high level of glucagon leads to severe hepatic mitochondrial dysfunction through increasing FOXO1. Hyperglucagonemia also contributes to the increased blood glucose level, which together with glucagon-induced hepatic mitochondrial dysfunction will result in other liver diseases (Fig. 8). Taken together, this study reveals a negative effect of glucagon on hepatic mitochondrial function in a FOXO1-dependent manner. This study further provides evidence that FOXO1, an important mediator in the glucagon signaling, is a potentially important therapeutic target for type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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