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UCP2 Regulates the Glucagon Response to Fasting and Starvation

Emma M. Allister,¹ Christine A. Robson-Doucette,¹ Kacey J. Prentice,¹ Alexandre B. Hardy,¹ Sobia Sultan,¹ Herbert Y. Gaisano,¹ Dong Kong,² Patrick Gilon,³ Pedro L. Herrera,⁴ Bradford B. Lowell,² and Michael B. Wheeler¹

Glucagon is important for maintaining euglycemia during fasting/starvation, and abnormal glucagon secretion is associated with type 1 and type 2 diabetes; however, the mechanisms of hypoglycemia-induced glucagon secretion are poorly understood. We previously demonstrated that global deletion of mitochondrial uncoupling protein 2 (UCP2^{-/-}) in mice impaired glucagon secretion from isolated islets. Therefore, UCP2 may contribute to the regulation of hypoglycemia-induced glucagon secretion, which is supported by our current finding that UCP2 expression is increased in nutrient-deprived murine and human islets. Further to this, we created α -cell-specific UCP2 knockout (UCP2AKO) mice, which we used to demonstrate that blood glucose recovery in response to hypoglycemia is impaired owing to attenuated glucagon secretion. UCP2-deleted α -cells have higher levels of intracellular reactive oxygen species (ROS) due to enhanced mitochondrial coupling, which translated into defective stimulus/secretion coupling. The effects of UCP2 deletion were mimicked by the UCP2 inhibitor genipin on both murine and human islets and also by application of exogenous ROS, confirming that changes in oxidative status and electrical activity directly reduce glucagon secretion. Therefore, α -cell UCP2 deletion perturbs the fasting/hypoglycemic glucagon response and shows that UCP2 is necessary for normal α -cell glucose sensing and the maintenance of euglycemia. *Diabetes* 62:1623–1633, 2013

Elevated basal glucagon levels and reduced hypoglycemia-induced glucagon secretion are underappreciated and poorly understood aspects of type 1 and type 2 diabetes (1–3). Although high plasma glucose normally inhibits glucagon secretion, it remains unclear whether this *in vivo* response is mediated by glucose sensing, neuronal modulation, paracrine/endocrine control, or a combination thereof (4–10). Uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, is expressed in pancreatic α -cells (11), and its expression can be induced in adipose tissue by a ketogenic diet (12), suggesting a role in the fasting response. While the precise physiological function of UCP2 in islet cells is still debated, it can mildly dissipate the proton motive force

generated during mitochondrial electron transport and limit ATP synthesis under certain conditions (13–15). Additionally, UCP2 can limit mitochondrial reactive oxygen species (ROS) production, which can alter associated signaling pathways and/or protect against oxidative stress (16–18). In β -cells, UCP2 deletion elicits only small changes in mitochondrial membrane potential ($\Delta\Psi_m$) with limited effect on ATP (18,19) but rather increases ROS production, which amplifies insulin secretion (18,20). α -Cells, like β -cells, have glucose-sensing machinery that center on K_{ATP} channel activity, cellular depolarization, and calcium influx, triggering exocytosis; however, unlike β -cells, they are electrically active and secretory at low glucose concentrations (5,21–24). UCP2 in α -cells could therefore be an important regulator of glucagon secretion via regulation of ATP production, plasma membrane potential, and ROS levels.

Previously, we showed that islets from mice globally lacking UCP2 (UCP2^{-/-}) displayed higher basal glucagon secretion and impaired low glucose-mediated glucagon secretion (11). Due to UCP2's wide expression profile in glucose-sensitive tissues, these changes in α -cell function in UCP2^{-/-} mice could be the result of β -cell and/or extra-pancreatic deletion. To decipher the role of UCP2 in α -cells and in the response to fasting, we created an α -cell-specific UCP2 knockout (UCP2AKO) deletion mouse model. These mice displayed reduced fasting plasma glucagon levels and impaired glucagon secretion, due in part to elevated ROS, enhanced glucose-induced hyperpolarization of the $\Delta\Psi_m$, and depolarization of plasma membrane potential. Therefore, we conclude that α -cell UCP2 plays a key role in the hypoglycemic response.

RESEARCH DESIGN AND METHODS

Animals. *LoxUCP2* mice on a 129/SV background (25) (Fig. 1A) were crossed for at least four generations to rat glucagon promoter-driven Cre recombinase (*Gcg-cre*) mice maintained on a C57BL/6 background (26). Male mice were genotyped using standard PCR (Supplementary Fig. 1A and Supplementary Table 1) and used for experiments at age 6–8 weeks. *Gcg-cre* mice were used as experimental controls, as *Gcg-cre* and floxed mice had similar phenotypes (Supplementary Fig. 2). For imaging experiments, *loxUCP2* mice were crossed to *Gcg-cre-ROSA26EYFP* mice (27) for at least four generations, producing mice with both α -cell-specific UCP2 deletion and expression of eYFP (UCP2AKO-YFP). All experiments were approved by the Animal Care Committee (University of Toronto) and animals handled according to the Canadian Council of Animal Care guidelines.

Human islets. Human islets from review board approved healthy donors were isolated using the Edmonton protocol (28,29) and provided by the Clinical Islet Laboratory or IsletCore (University of Alberta, Edmonton, Canada). Islets were picked into RPMI media with 10% FBS and cultured overnight before experimentation.

Antibodies and reagents. The monoclonal Cre antibody was from Covance (San Diego, CA) and the polyclonal UCP2 antibody from Everest Biotech (Oxfordshire, U.K.). Guinea pig anti-swine insulin (Dako Canada), rabbit anti-human glucagon (Dako Canada), and fluorescein isothiocyanate- and cyanine 5-labeled secondary antibodies (Jackson ImmunoResearch) were also used.

From the ¹Department of Physiology, University of Toronto, Toronto, Ontario, Canada; the ²Division of Endocrinology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; the ³Pôle d'endocrinologie, diabète et nutrition, Institut de recherche expérimentale et clinique, Université catholique de Louvain, Brussels, Belgium; and the ⁴Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland.

Corresponding author: Michael B. Wheeler, michael.wheeler@utoronto.ca. Received 20 July 2012 and accepted 19 December 2012.

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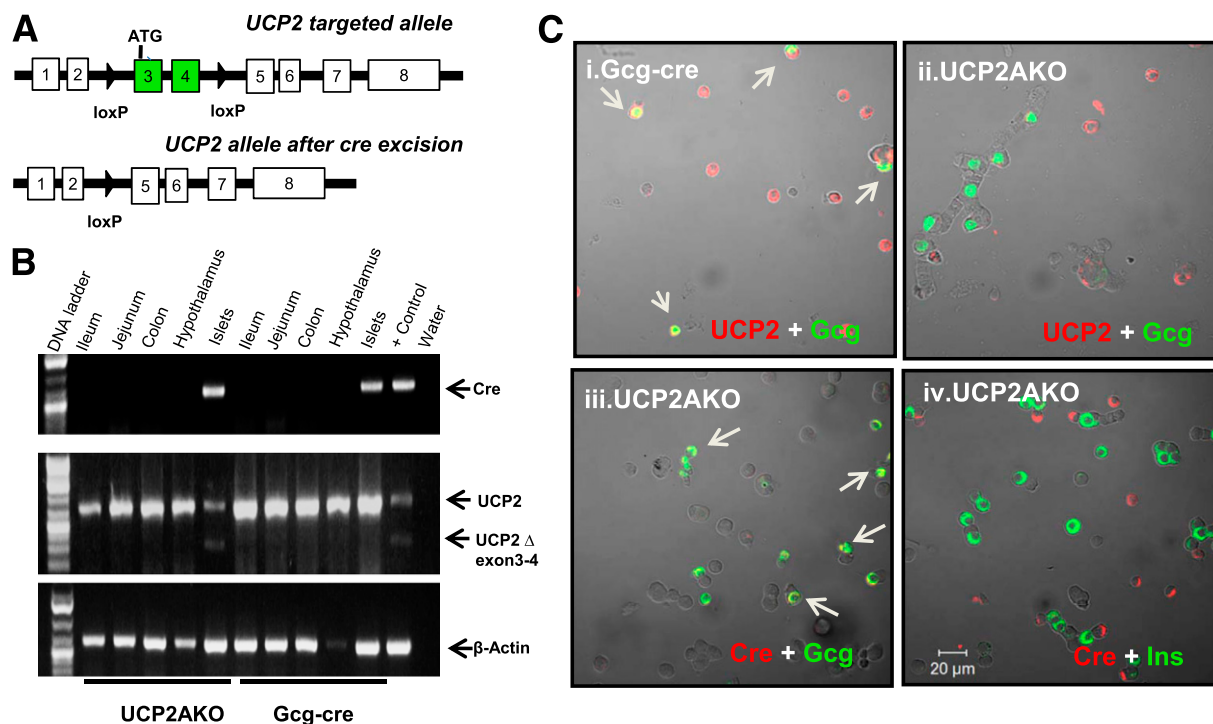


FIG. 1. UCP2 is efficiently deleted specifically from islet α -cells of UCP2AKO mice. **A:** Schematic diagram of the UCP2-targeting construct. **B:** Cre and UCP2 tissue expression measured by standard PCR. **C:** Immunostaining for UCP2 protein (red) and glucagon (green) in dispersed islet cells from Gcg-cre and UCP2AKO mice. UCP2AKO islet cells were also stained for Cre (red) and glucagon (green) or insulin (green). Colocalization is indicated by yellow and arrows. $N = 3$.

Rhodamine 123, H_2O_2 , and RedTaq polymerase were from Sigma-Aldrich. Genipin was from Wako.

Immunofluorescent staining. Dispersed islet cells plated onto glass coverslips were fixed, stained with antibodies, and fluorescence visualized using a laser-scanning confocal microscope (Zeiss 510 LSM) (11).

Plasma hormone concentration and tolerance tests

Insulin tolerance tests. After a 4-h fast, insulin (1.75 IU/kg body wt) was injected intraperitoneally and blood glucose levels were measured at 0, 15, 30, 60, and 120 min. Plasma glucagon was measured at 0 and 30 min by RIA (Millipore).

Oral glucose tolerance test. After a 4-h fast, glucose (3 g/kg body wt) was gavaged and blood glucose levels were measured at 0, 5, 10, 15, 30, 60, and 120 min. Plasma active glucagon-like peptide (GLP)-1 (Meso Scale Discovery, Gaithersburg, MD) was measured at 0 and 10 min.

Fasting/re-feeding hepatic gluconeogenic gene expression, hepatic glycogen, and plasma metabolites. Plasma glucose, glucagon, insulin, and corticosterone (MP Biomedicals, Irvine, CA) were measured in fed, 24-h fasted, and 12-h re-fed mice. Mice were killed under fasting or re-fed conditions, and liver RNA was extracted using Trizol (Invitrogen, Canada) for measurement of gluconeogenic and glycolytic enzyme genes (Supplementary Table 1). The glycogen content of livers from 24-h fasted and 12-h re-fed mice was extracted as previously described (30) and measured using a colorimetric kit (Biovision). The relative concentrations of plasma metabolites were measured in 100 μ L plasma from fed and 24-h fasted mice by mass spectrometry (Metabolon). Sample preparation, metabolite identification, data normalization, and statistical analysis were performed as previously described (31).

Islet glucose-inhibited glucagon secretion. Fresh isolated islets (mouse or human) were handpicked into 11 mmol/L glucose RPMI media with 10% FBS and preincubated for 1 h with or without genipin (50 μ mol/L) or H_2O_2 (16 μ mol/L), which remained throughout the protocol. Fifteen islets per condition were preincubated for 15 min with 20 mmol/L glucose Krebs-Ringer buffer (KRB) (11) before incubation with either high- (20 mmol/L) or low- (1 mmol/L) glucose KRB for 1 h. Glucagon concentrations were determined by RIA (Millipore) and normalized to DNA content.

Mitochondrial membrane potential ($\Delta\Psi_m$). α -Cells were identified by YFP fluorescence before rhodamine 123 (25 μ g/mL; 10 min) loading in 1 mmol/L glucose KRB on the microscope stage. The cells were then washed and imaged in 1 and 20 mmol/L glucose KRB. NaN_3 (5 mmol/L) was added to fully depolarize the $\Delta\Psi_m$ (11).

Islet ATP content and intracellular calcium. Islet ATP content was measured as previously described using 15 islets per condition (18). Cells were preincubated with or without H_2O_2 (16 μ mol/L) or genipin (50 μ mol/L) for 1 h in 11 mmol/L glucose RPMI and then loaded with 2 μ mol/L Fura-2 AM (Molecular Probes; Invitrogen) in 20 mmol/L glucose KRB for 45 min before imaging of cytosolic calcium in YFP $^+$ α -cells (32).

Electrophysiology. Electrophysiological recordings of K_{ATP} currents in YFP $^+$ α -cells were performed as previously described (33). Plasma membrane potential was measured using a perforated patch technique in whole-cell configuration. The extracellular solution for membrane potential measurements contained (in millimoles per liter) 140 NaCl, 3.6 KCl, 0.5 $MgSO_4$, 1.5 $CaCl_2$, 10 HEPES, 0.5 NaH_2PO_4 , 5 $NaHCO_3$ (pH 7.4 with NaOH), and 20 glucose. The intracellular solution contained (in millimoles per liter unless otherwise indicated) 76 K_2SO_4 , 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES (pH 7.35 with KOH), and 60 μ g/mL gramicidin (34). Cells displaying input resistance <25 M Ω were studied. Average membrane potentials were calculated across 40 s of steady-state recordings.

ROS accumulation in isolated islets and its effect on glucagon secretion. The levels of superoxide and H_2O_2 were determined using MitosoxRed and 2',7'-dichlorodihydro fluorescein diacetate (CM- H_2 -DCFDA), respectively (Molecular Probes; Invitrogen) (35). Fresh isolated islets were preincubated with or without H_2O_2 (16 μ mol/L) or genipin (50 μ mol/L) in 11 mmol/L glucose RPMI for 1 h, followed by 1 mmol/L glucose KRB with or without treatments for 1 h before measurement of superoxide or H_2O_2 levels. Alternatively, islets were cultured overnight in 11 mmol/L glucose RPMI and then H_2O_2 levels measured with CM- H_2 -DCFDA.

Quantitative real-time PCR. Quantitative PCR was performed as previously described (18,32). Primers are listed in Supplementary Table 1.

Statistics. Statistical significance was assessed using either the Student t test or a two-way ANOVA for repeated measures followed by a Bonferroni posttest comparison using GraphPad Prism 4. $P < 0.05$ was considered significant. All data are means \pm SEM.

RESULTS

Deletion of α -cell UCP2 impairs glucagon secretion in response to hypoglycemia. Truncated UCP2, indicative of exon 3 and 4 deletion, was observed only in islets and not in the intestine or hypothalamus of UCP2AKO mice

(Fig. 1B). Cre (measured by eYFP staining) was expressed in $72 \pm 10\%$ glucagon-positive cells (Supplementary Fig. 1B) suggesting efficient deletion. Immunofluorescent staining of dispersed UCP2AKO islets showed cre protein only in α -cells (Fig. 1C), and specific deletion of UCP2 from UCP2AKO α -cells was confirmed by the absence of UCP2 only in glucagon-positive cells (Fig. 1C). Cre (YFP staining) was not expressed in the nucleus of the solitary tract or the distal ileum, reinforcing the specificity of the glucagon promoter for α -cells (Supplementary Fig. 3).

UCP2AKO and Gcg-cre mice displayed similar insulin sensitivities, as the rate and magnitude of blood glucose reduction during an insulin tolerance test were equal (Fig. 2A); however, the rate of blood glucose recovery was slower in UCP2AKO mice, resulting in significantly lower blood glucose at 120 min (Fig. 2A). This correlated with lower glucagon levels 30 min after insulin injection (just before blood glucose levels increased) (Fig. 2B), suggesting that UCP2AKO mice have impaired hypoglycemia-induced glucagon secretion.

Mice lacking glucagon receptors are more glucose tolerant compared with wild-type mice (36). Therefore, we tested whether UCP2AKO mice that display impaired glucagon secretion were more glucose tolerant. An oral glucose tolerance test showed that UCP2AKO mice had similar glucose tolerance to Gcg-cre mice (Fig. 3A) and secreted the same amount of active GLP-1 into plasma (Fig. 3B).

α -Cell UCP2 deficiency impairs hepatic gluconeogenic gene expression and causes a switch of fatty acid usage in response to a prolonged fast. To assess the effect of impaired glucagon secretion in UCP2AKO mice, we examined the effect of prolonged fasting/re-feeding on glucose homeostasis, hepatic gluconeogenesis, and glycogenolysis. Although blood glucose (Fig. 4A) and plasma insulin (Fig. 4C) were similarly decreased upon fasting, the UCP2AKO mice displayed reduced fasting plasma glucagon (Fig. 4B). The difference in plasma glucagon levels was not due to impaired secretion of corticosterone, as the levels were similarly increased upon fasting (Supplementary Fig. 4A).

The liver is the major target organ for glucagon in vivo, and so we explored the effect of prolonged fasting/re-feeding on hepatic glucose metabolism genes and glycogen content. After fasting, UCP2AKO livers displayed impaired induction of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*Pepck*) (Fig. 4D) and glucose-6-phosphatase (*G6pc*) (Fig. 4E). Conversely, the glycolytic gene, glucokinase

(*Gck*), was significantly higher (Fig. 4G). The expression of fructose 1,6-bisphosphatase (*Fbp1*) decreased and fatty acid synthase (*Fasn*) increased similarly upon re-feeding (Fig. 4F and H), suggesting an intact response for these genes. The reduced gluconeogenic gene expression in the UCP2AKO mice supports their impaired glucagon secretion in response to hypoglycemia. Glycogen levels were 2.5-fold higher in UCP2AKO livers upon fasting (Fig. 4I), also reflecting their lower glucagon levels.

A targeted mass spectrometry approach examining multiple metabolic pathways was used to determine the effect of reduced glucagon secretion and the impaired hepatic gluconeogenic response to fasting on plasma metabolites. Of the 307 identified biochemicals, 157 were significantly changed upon fasting and 63 showed a fed-state, genotype interaction ($P < 0.05$). Levels of the ketone body 3-hydroxybutyrate normally increase upon fasting, and the response of this metabolite was intact in UCP2AKO mice (Supplementary Fig. 4B). Interestingly, several abnormalities in lipid metabolism products were observed in UCP2AKO mice. The levels of medium chain-length fatty acids (MCFAs) were similar in fed plasma; however, their concentration decreased in UCP2AKO and increased in Gcg-cre mice upon fasting (Fig. 4J and Supplementary Fig. 4C), suggesting that UCP2AKO mice may preferentially use MCFAs as an energy source under these conditions.

UCP2 expression is increased after nutrient depletion in islets, and α -cell UCP2 deficiency/inhibition impairs glucagon secretion from isolated islets. Human and mouse islets deprived of nutrients (low serum and low glucose) showed similar induction of glucagon and UCP2 expression, highlighting the potential importance of UCP2 during fasting (Fig. 5A and B). We therefore assessed the ability of fresh-isolated UCP2AKO mouse islets to secrete glucagon under low glucose conditions. When conditions were switched from high to low glucose, glucagon secretion was significantly stimulated in Gcg-cre and UCP2AKO islets ($P < 0.001$) (Fig. 5D); however, the amount secreted by UCP2AKO islets was 52 and 31% lower under high and low glucose concentrations, respectively. Total glucagon content and islet morphology were similar in the two genotypes, suggesting that the reduced secretion from UCP2AKO islets was not due to reduced glucagon content or α -cell number (data not shown).

Gcg-cre islets exposed to genipin, a compound known to inhibit UCP2 (37), secreted significantly less glucagon in the presence of low glucose, and this level was similar

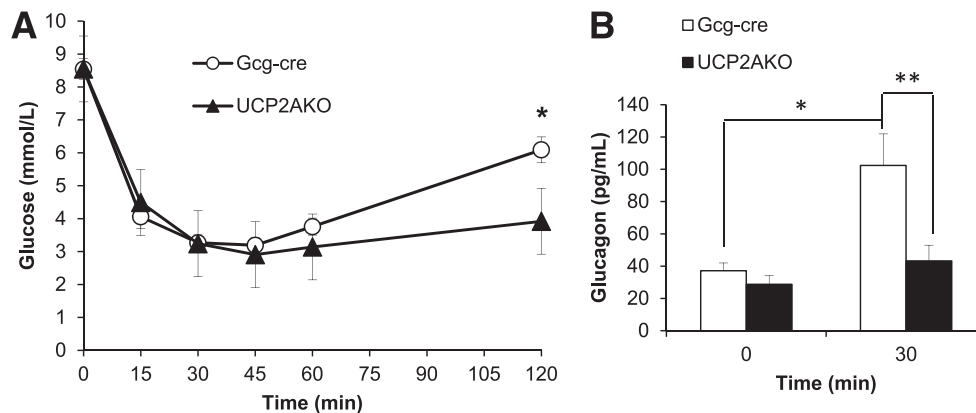


FIG. 2. α -Cell UCP2 deletion reduces glucagon secretion in vivo. **A:** Blood glucose during an intraperitoneal insulin tolerance test (ipITT). $*P < 0.05$. $N = 12$ –15. **B:** Plasma glucagon levels at 0 and 30 min during the ipITT. $N = 6$ –8 mice/group. $*P < 0.05$; $**P < 0.01$.

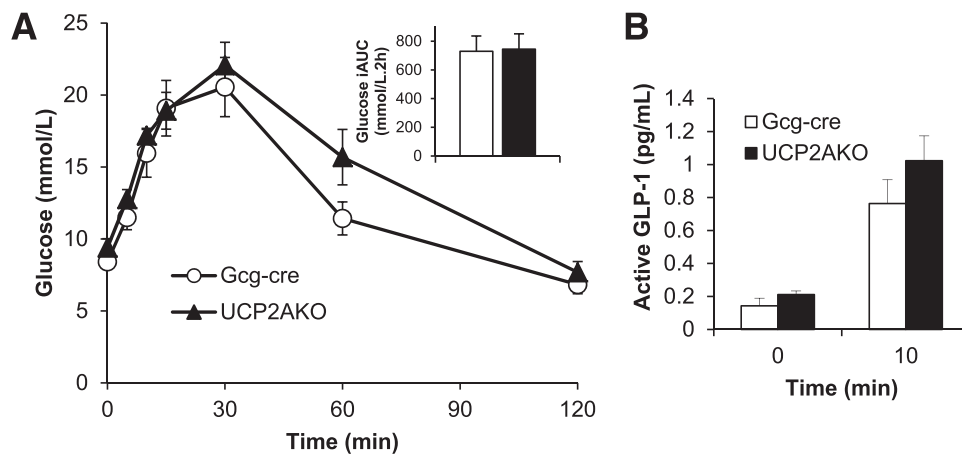


FIG. 3. UCP2AKO mice display normal glucose tolerance and GLP-1 secretion. *A* and *B*: Oral glucose tolerance test; mice were gavaged with 3 g/kg glucose after a 4-h fast ($N = 7-8$ mice/genotype). Blood glucose (*A*) and plasma active GLP-1 (*B*) were measured. The inset panel in *A* shows the incremental area under the glucose curve (iAUC).

to UCP2AKO islets in the presence of low glucose alone (Fig. 5E). Conversely, preincubation of UCP2AKO islets with genipin did not alter glucagon secretion (Fig. 5E). For assessment of the interspecies effect of UCP2 inhibition on glucagon secretion, human islets were also exposed to genipin. They secreted significantly less glucagon under low glucose conditions, which was similar to high glucose levels alone (Fig. 5C). Therefore, acute inhibition of UCP2 with genipin reduces glucagon secretion under stimulatory low glucose conditions.

α -Cell UCP2 deficiency increases glucose-induced $\Delta\Psi_m$ hyperpolarization, which leads to altered oxidative status and impaired glucagon secretion. To determine the contribution of UCP2 to mitochondrial coupling in α -cells, we measured changes in glucose-induced $\Delta\Psi_m$ hyperpolarization (Fig. 6A). Basal (1 mmol/L glucose) $\Delta\Psi_m$ was similar, but UCP2AKO-YFP α -cells displayed significantly greater glucose-induced hyperpolarization of $\Delta\Psi_m$ (Fig. 6B).

Even small increases in $\Delta\Psi_m$ can translate into significantly large increases in mitochondrial ROS production, and recently ROS signaling has emerged as an important regulator of insulin secretion (18,20,38). In contrast, chronic oxidative stress can impair secretion (17,35). As such, we compared the levels of intracellular ROS (H_2O_2 and mitochondrial superoxide) in fresh-isolated islets (to correlate with glucagon secretion studies [Fig. 5D]) and H_2O_2 in cultured islets. No difference in the amount of H_2O_2 but significantly more mitochondrial superoxide was observed in fresh-isolated UCP2AKO islets (Fig. 6C and D). Application of genipin increased H_2O_2 levels 1.37-fold in Gcg-cre islets only ($P < 0.01$) and increased both H_2O_2 and superoxide levels in human islets (Fig. 6H and I). Therefore, UCP2 deficiency in mouse α -cells results in superoxide buildup and an inability to convert superoxide to H_2O_2 . However, after overnight culture, UCP2AKO islets displayed ~ 1.7 -fold greater intracellular H_2O_2 (Fig. 6E). Cultured Gcg-cre islets appeared to recover from the stress of isolation, as H_2O_2 levels were lower than in fresh-isolated islets (Fig. 6C), suggesting that ROS production is increased in UCP2AKO islets and they experience a chronically altered oxidative status. Whole-islet data were confirmed in dispersed, cultured α -cells, as overall ROS levels were higher in UCP2AKO α -cells (Supplementary Fig. 5). In

addition, treatment of Gcg-cre α -cells with genipin increased ROS to a similar level as UCP2AKO α -cells, whereas genipin had no further effect on UCP2AKO α -cells (Supplementary Fig. 5).

Intracellular ROS levels were correlated with expression of several antioxidant-related genes in fresh isolated whole islets. The expression of superoxide dismutase 2 (*Sod2*) was 88% lower in UCP2AKO islets (Fig. 6F), consistent with their inability to convert superoxide to H_2O_2 , whereas the H_2O_2 -scavenging glutathione peroxidase (*Gpx*) and catalase (*Cat*) and the cytoprotective gene, Heme Oxygenase 1 (*Ho-1*), were unchanged (Supplementary Fig. 6A).

To determine whether increased ROS contribute to impaired glucagon secretion from UCP2AKO islets, we increased intracellular ROS with exogenous H_2O_2 application (Fig. 6C and D). Gcg-cre islets preincubated with H_2O_2 secreted significantly more glucagon under high-glucose and less under low glucose conditions (Fig. 6G). The increased glucagon secretion in the presence of high glucose plus H_2O_2 occurred despite significantly greater insulin secretion (Supplementary Fig. 6C). The addition of H_2O_2 to UCP2AKO islets did not alter glucagon secretion (Fig. 6G). Similarly, the addition of H_2O_2 had no effect on glucose-mediated insulin secretion from UCP2AKO islets (Supplementary Fig. 6C). Therefore, elevated ROS levels in control islets impaired glucagon secretion similarly to UCP2AKO islets, which experience endogenously higher ROS levels and altered oxidative status.

Impaired glucagon secretion from UCP2-deficient α -cells correlates with enhanced depolarization of plasma membrane potential and reduced calcium. Metabolism of glucose and further closure of K_{ATP} channels reduce glucagon secretion (24). Therefore, we measured K_{ATP} current and plasma membrane potential. K_{ATP} currents in Gcg-cre-YFP and UCP2AKO-YFP α -cells could be similarly activated and inhibited by diazoxide and tolbutamide, respectively (Supplementary Fig. 7A). However, despite a similar magnitude of change in plasma membrane potential when switched from high to low glucose, the plasma membrane was more depolarized in UCP2AKO-YFP α -cells under both conditions (Fig. 7A and B). Preincubation of Gcg-cre-YFP α -cells with high glucose plus genipin increased (depolarized) plasma membrane potential

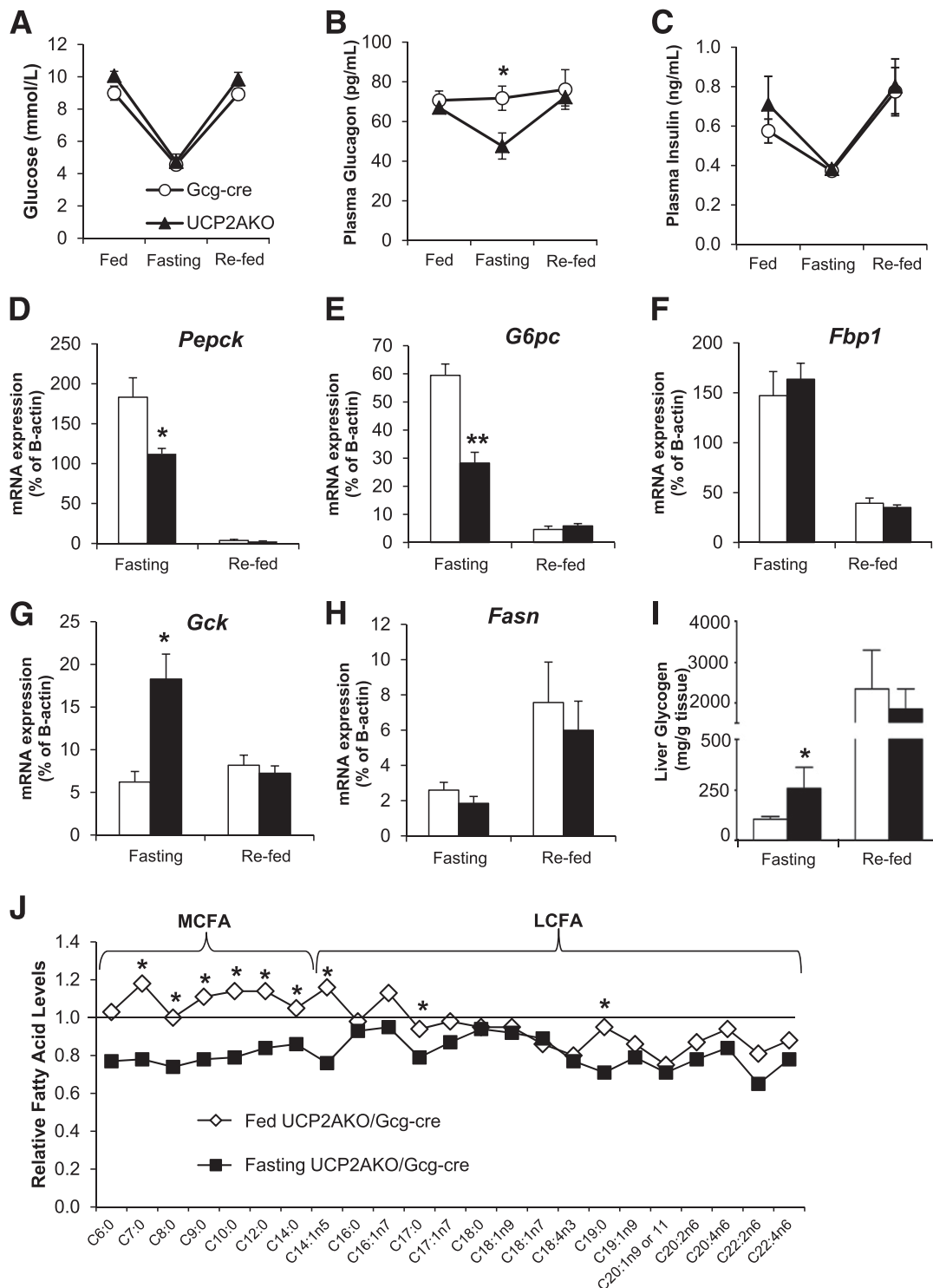


FIG. 4. α -Cell UCP2 deletion reduces the gluconeogenic response of the liver and switches fatty acid usage during a prolonged fast. Blood glucose (A), plasma glucagon (B), and plasma insulin (C) measured in fed, 24-h fasted (fasting), and 12-h re-fed mice. * $P < 0.05$. D–H: Hepatic expression of the enzymes *Pepck* (D), *G6pc* (E), *Fbp1* (F), *Gck* (G), and *Fasn* (H) genes. $N = 4$ –8/group. * $P < 0.05$ or ** $P < 0.01$ compared with Gcg-cre. I: Hepatic glycogen levels. $N = 4$ –6 mice/condition. * $P < 0.05$. J: Metabolic analyses of MCFA and long-chain fatty acid (LCFA) plasma levels. Data are relative to Gcg-cre levels. $N = 8$ mice/genotype. * $P < 0.05$ genotype/fed-state interaction.

but had no further effect in UCP2AKO-YFP α -cells (Fig. 7C). Therefore, the absence of UCP2 in α -cells shifts the operating range of plasma membrane potentials, which might explain the impaired glucagon secretion.

The more depolarized plasma membrane potential in the presence of high glucose suggests that α -cell UCP2

deficiency may increase ATP levels and close/inactivate K_{ATP} channels. There was a trend toward increased ATP content in UCP2AKO islets under high glucose conditions (Supplementary Fig. 7B); however, any effect of α -cell UCP2 deletion may be masked by β -cell ATP content. Therefore, we determined whether the K_{ATP} channel

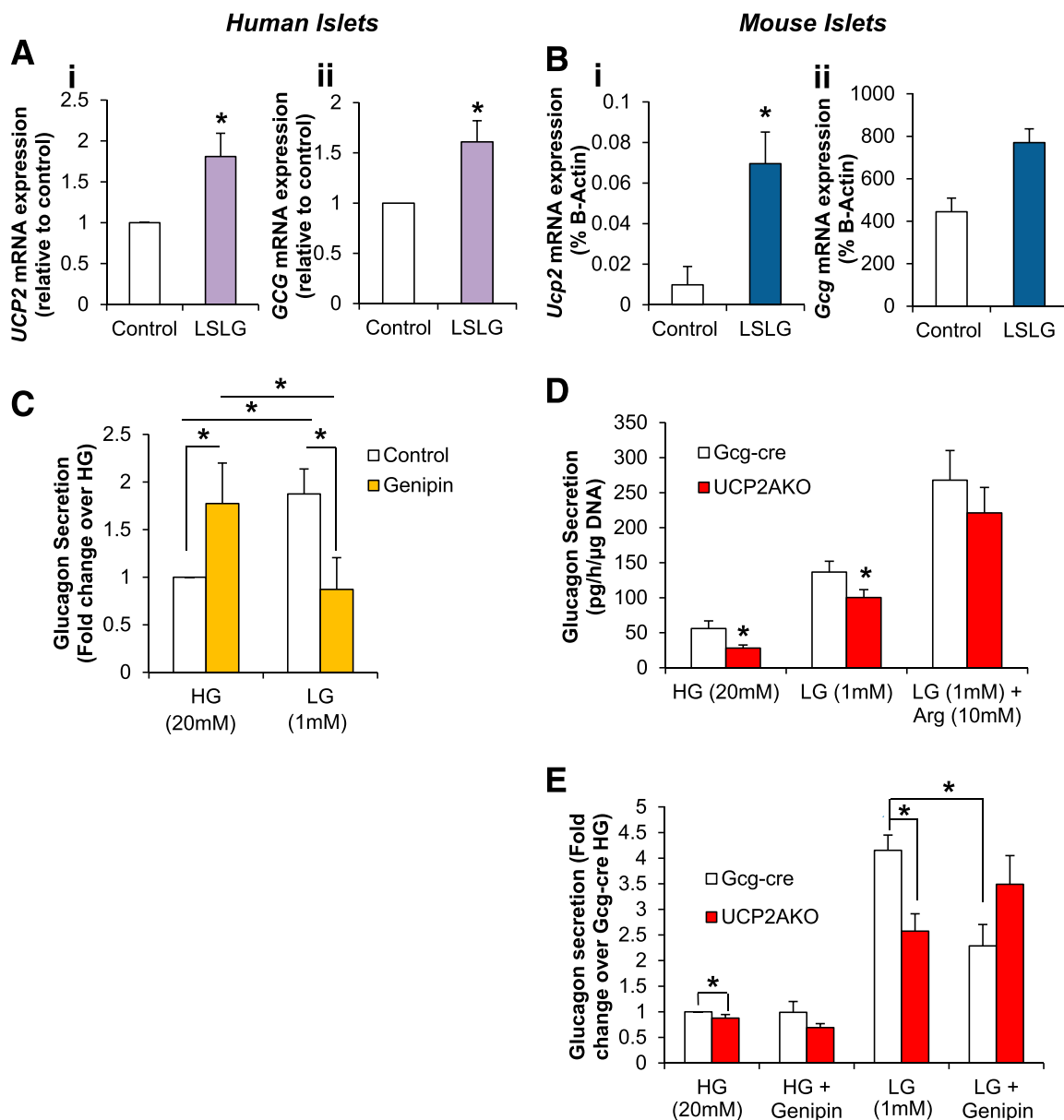


FIG. 5. UCP2 expression is increased after nutrient depletion and glucagon secretion from UCP2AKO islets was impaired. Human (*left panel*) and mouse (*right panel*). **A:** *UCP2* (i) and *GCG* (ii) expression in human islets exposed to low serum (0.01% FBS) and low glucose (2.8 mmol/L) RPMI (low serum and low glucose [LSLG]) for 6 h. $N = 6$ individual donors. **B:** *Ucp2* (i) and *Gcg* (ii) expression in control CD1 mouse islets exposed to LSLG for 6 h. $N = 4$ –5/condition. **C:** Glucose-inhibited glucagon secretion from human islets preincubated with or without genipin (50 μ mol/L) during a half-hour static incubation. $N = 6$ –7 donors. **D:** Glucose-inhibited glucagon secretion was measured from fresh-isolated islets incubated with high glucose (HG) (20 mmol/L), low glucose (LG) (1 mmol/L), or low glucose plus arginine (LG + Arg) (10 mmol/L). $N = 8$ –9 mice/genotype. **E:** Glucose-inhibited glucagon secretion measured from islets preincubated with or without genipin (50 μ mol/L). $N = 7$ –9 mice/genotype. All mouse data are normalized to DNA, but the human data are per islet. * $P < 0.05$.

opener, diazoxide, could overcome the impaired secretion. Under high glucose conditions, the addition of low-dose diazoxide (1 μ mol/L) increased glucagon secretion from UCP2AKO islets to a level similar to that in Gcg-cre islets without diazoxide; however, the UCP2AKO islets were unable to reach a level of secretion as high as that in Gcg-cre islets in the presence of 1 μ mol/L diazoxide (Fig. 7D). Conversely, under low glucose conditions, low-dose diazoxide increased glucagon secretion from UCP2AKO islets to a level similar to that in Gcg-cre islets in the absence of diazoxide. The addition of more diazoxide dose-dependently decreased secretion (Fig. 7D), which was similar to previous observations (24). Therefore, under low glucose conditions

opening K_{ATP} channels can correct the glucagon secretion defect observed in UCP2AKO islets.

Influx of calcium is required for low glucose-mediated exocytosis of glucagon (39), and thus we examined changes in cytosolic calcium. The amount of intracellular calcium under high and low glucose conditions was reduced in YFP-UCP2AKO α -cells (Fig. 7F). Pretreatment of YFP-Gcg-cre cells with genipin also significantly reduced intracellular calcium levels, whereas H_2O_2 had no effect (Fig. 7F). In addition, intracellular calcium in UCP2AKO α -cells oscillated faster under low glucose conditions (Supplementary Fig. 8E). Reduced cytosolic calcium in UCP2AKO α -cells reflects an impaired ability to secrete glucagon.

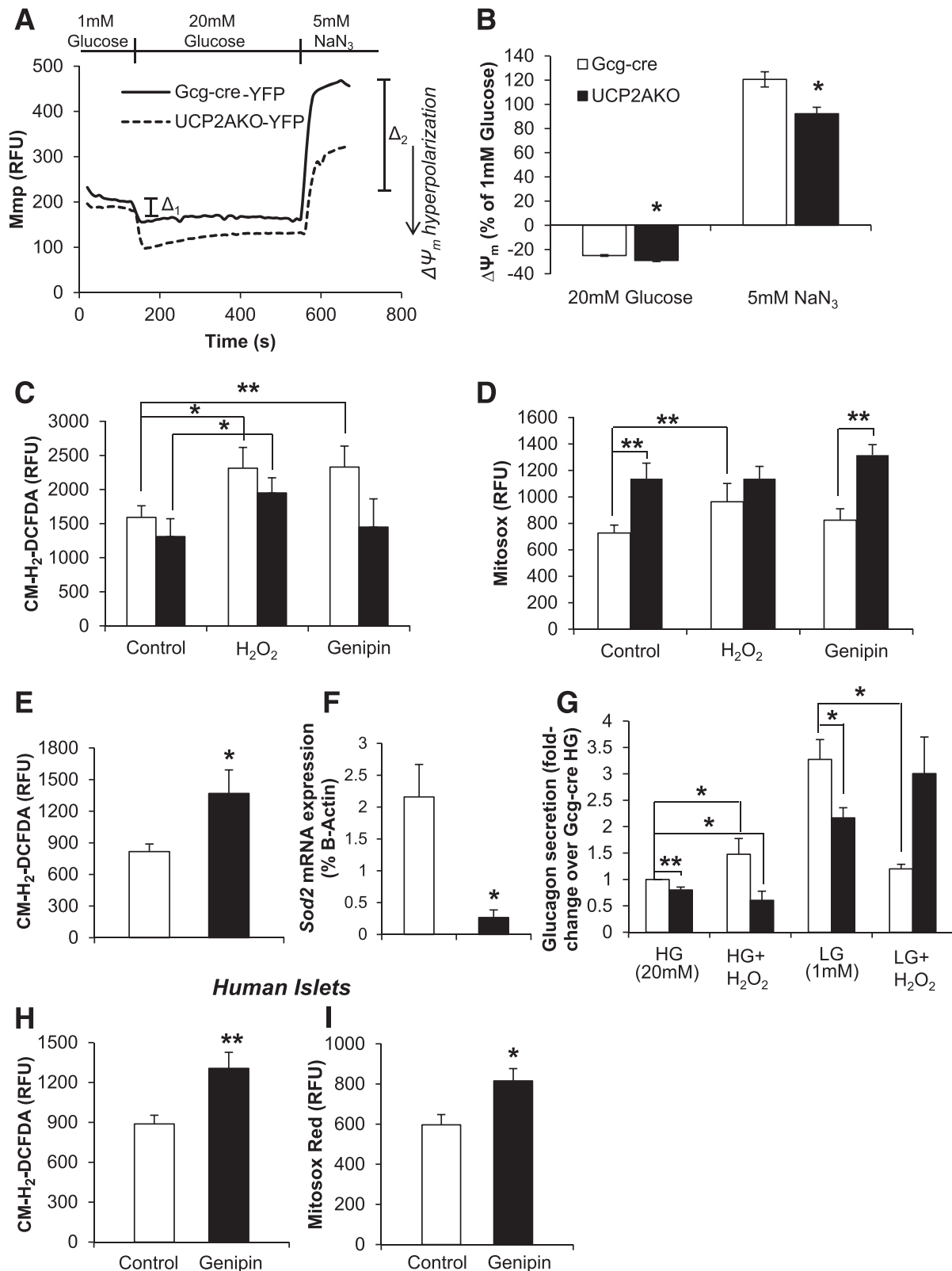


FIG. 6. UCP2AKO α -cells display enhanced hyperpolarization of $\Delta\Psi_m$ and increased superoxide levels. **A:** Representative traces of $\Delta\Psi_m$ (Mmp) relative fluorescent unit (RFU) measurements in YFP⁺ α -cells. **B:** $\Delta\Psi_m$ (Mmp) was measured in high glucose (20 mmol/L) (Δ_1) and normalized to basal fluorescence in the presence of low glucose (1 mmol/L) (Δ_2) and NaN₃ (5 mmol/L) (Δ_3) and normalized to basal fluorescence in the presence of low glucose (1 mmol/L). $N = 28$ –29 cells from 6 mice/genotype. $*P < 0.05$. **C** and **D:** Fresh-isolated islets were cultured with or without H₂O₂ (16 μ mol/L) or genipin (50 μ mol/L) before measurement of H₂O₂ (CM-H₂-DCFDA) (**C**) or superoxide (MitoxoxRed) (**D**). $N = 20$ –28 islets, 6–8 mice/genotype. $*P < 0.05$, $**P < 0.01$. **E:** Islets were cultured overnight, and then intracellular H₂O₂ (CM-H₂-DCFDA) was measured. $N = 19$ –27 islets, 5–7 mice/genotype. $*P < 0.05$. **F:** Islet *Sod2* expression. $N = 4$ –5 mice/genotype. $*P < 0.05$. **G:** Glucose-inhibited glucagon secretion with or without H₂O₂ (16 μ mol/L). Secretion is per microgram of DNA, normalized to Gcg-cre high glucose (HG) conditions. LG, low glucose. $N = 3$ –11 mice/genotype. $*P < 0.05$, $**P < 0.01$. **H** and **I:** Human islets were incubated with 50 μ mol/L genipin for 2 h before measurement of H₂O₂ (CM-H₂-DCFDA) (**H**) and superoxide (MitoxoxRed) (**I**) levels. $N = 4$ individual donors. $*P < 0.05$, $**P < 0.01$.

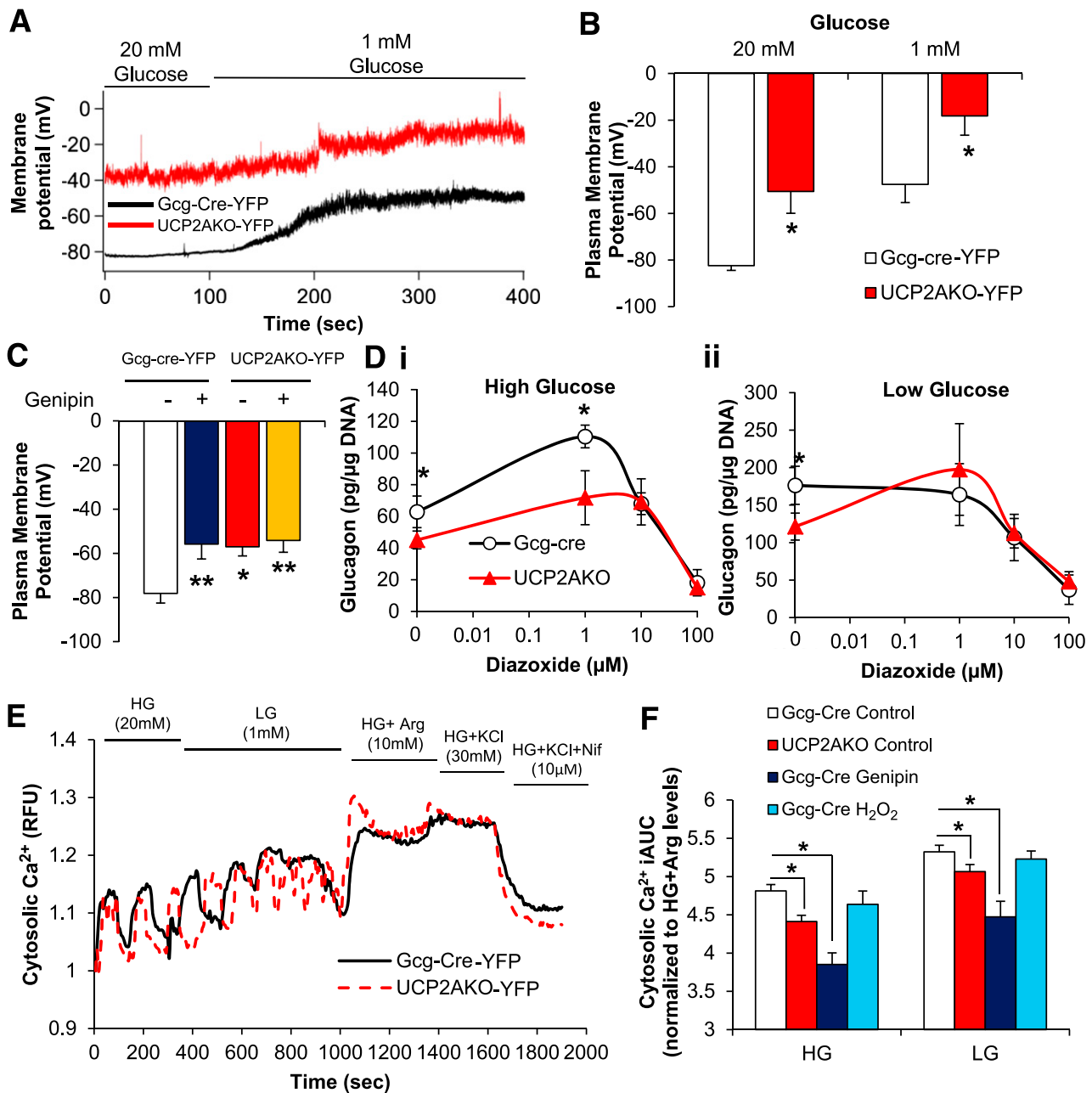


FIG. 7. UCP2AKO α -cells have more depolarized plasma membranes and reduced intracellular calcium. **A:** Representative traces of plasma membrane potential in YFP⁺ α -cells incubated in high (20 mmol/L) and low (1 mmol/L) glucose. **B:** Average membrane potential in YFP⁺ α -cells. $N = 5$ –8 cells, 3 mice/genotype. $*P < 0.05$. **C:** Average plasma membrane potential measured in YFP⁺ α -cells ($N = 8$ –13) with or without genipin (50 μ mol/L) ($N = 5$ cells), and at least 3 mice/genotype. $*P < 0.05$, $**P < 0.01$. **D:** Glucose-inhibited glucagon secretion from islets incubated with or without diazoxide (1, 10, and 100 μ mol/L) in the presence of high glucose (20 mmol/L) (i) or low glucose (1 mmol/L) (ii). $*P < 0.05$. $N = 3$ mice/genotype. **E:** Representative traces of cytosolic calcium uptake in YFP⁺ α -cells switched from high glucose (HG) (20 mmol/L) to low glucose (LG) (1 mmol/L) and then in the presence of arginine (Arg) (10 mmol/L), KCl (30 mmol/L), and the L-type calcium blocker nifedipine (Nif) (10 μ mol/L). **F:** Incremental area under the curve (iAUC) for cytosolic calcium, normalized to high glucose plus arginine levels. Representative traces are shown in Supplementary Fig. 8. $*P < 0.05$. $N = 19$ –40 cells/treatment, 3 mice/genotype.

DISCUSSION

α -Cells secrete glucagon in response to low blood glucose and are inhibited when blood glucose levels rise (24). We now show that UCP2 expression is increased in islets deprived of nutrients, α -cell UCP2 is necessary for appropriate glucagon secretion, and the absence of UCP2 impairs α -cell function. UCP2AKO mice displayed impaired glucagon secretion in vivo, which inhibited the hepatic response to hypoglycemia (40), including reduced expression of the hepatic gluconeogenic genes, *Pepck* and *G6pc*, and higher glycogen levels. Reduced fasting levels of

plasma MCFAs in UCP2AKO mice suggest compensation for impaired hepatic gluconeogenesis and increased usage of these fatty acids during hypoglycemia. MCFAs are absorbed directly through the portal vein, making them a direct energy source for the liver (41). MCFAs also produce more ketones per unit of energy than long-chain fatty acids, which could provide crucial energy for the brain during nutrient depletion and when gluconeogenesis is impaired, such as in fasted UCP2AKO mice. Similarly, mice globally lacking glucagon receptors demonstrate impaired activation of hepatic gluconeogenic genes and

enhanced lipogenesis, yet have improved glucose tolerance (36,42). Interestingly, UCP2AKO mice, similar to adult mice with 98% α -cell ablation (43), displayed normal glucose tolerance. Therefore, we show that appropriate glucagon secretion is required for normal hepatic function during fasting and that α -cell UCP2 deficiency disrupts this process, solidifying the role of UCP2 in the α -cell response to hypoglycemia.

The amount of glucagon secreted from UCP2AKO islets under stimulatory conditions was the same as that secreted from Gcg-cre islets under inhibitory conditions. Preincubation of Gcg-cre islets with the UCP2 inhibitor genipin reduced low glucose-mediated glucagon secretion by a magnitude similar to that in UCP2 deletion, whereas genipin did not further reduce glucagon secretion from UCP2AKO islets, suggesting its relative specificity of action for UCP2. Interestingly, the glucagon-lowering effect of genipin was not isolated to mouse islets, as it also significantly impaired low glucose-mediated glucagon secretion from human islets. Insulin released from β -cells lowers glucagon secretion via paracrine inhibition (5,7,9); however, the reduced glucagon secretion from UCP2AKO islets was not explained by this mechanism, as insulin secretion was similar from Gcg-cre and UCP2AKO cultured islets (Supplementary Fig. 6B). Therefore, the presence of UCP2 in α -cells is directly required for appropriate glucagon release.

The α -cell secretes glucagon within a narrow range of plasma membrane potentials (24) and is sensitive to small changes in ATP concentration (22), and it is suggested that glucose metabolism does not accelerate ATP synthesis in

this cell type (44). We previously showed that UCP2 is expressed at higher levels in α compared with clonal β -cell lines (11). Thus, high expression of UCP2 may normally limit ATP production in α -cells via an uncoupling role. Furthermore, small interfering RNA knockdown of UCP2 in clonal α -cells increased ATP levels (11). Although no difference in ATP concentrations in UCP2AKO versus Gcg-cre islets was detected, the increased glucose-induced hyperpolarization of $\Delta\Psi_m$ and more depolarized plasma membrane of UCP2AKO α -cells suggest increased ATP synthesis. Recent studies show that depolarization of α -cells inhibits the activity of Na^+ and Ca^{2+} channels resulting in cessation of glucagon secretion (22,24), and we show reduced intracellular calcium levels in UCP2AKO α -cells under both high and low glucose conditions. Similarly, exposure of Gcg-cre α -cells to genipin depolarized the plasma membrane and reduced calcium entry. As such, we propose that UCP2AKO α -cells have more closed K_{ATP} channels, limiting their glucagon secretion capacity (24,45). It was no surprise that the addition of low-dose diazoxide, which opens the K_{ATP} channel, corrected this impairment under low glucose conditions. Conversely, in the presence of high glucose, low-dose diazoxide increased glucagon secretion from UCP2AKO islets to a level similar to basal secretion from Gcg-cre islets, yet further addition of diazoxide did not elevate secretion from UCP2AKO islets. These data suggest that altered membrane potential is not the only factor limiting glucagon secretion from UCP2AKO islets under high glucose conditions.

It has been proposed that generation of low-level ROS, likely H_2O_2 , amplifies insulin secretion (20,46), and recently,

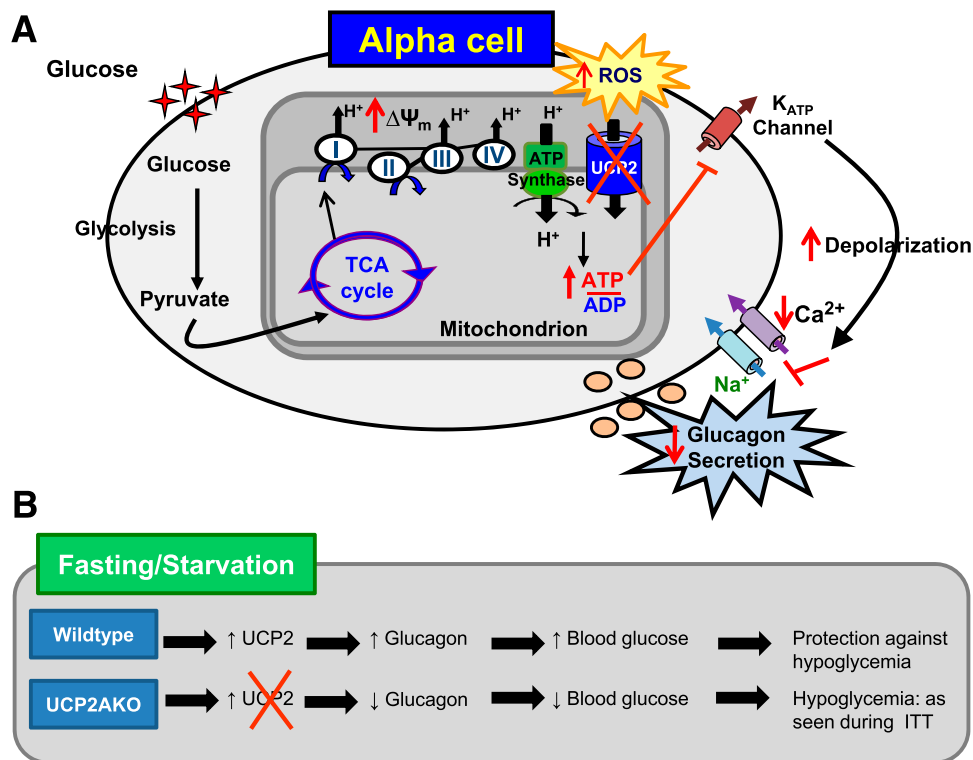


FIG. 8. UCP2 is required for normal glucagon secretion in response to hypoglycemia. **A:** UCP2AKO islets displayed increased glucose-induced hyperpolarization of $\Delta\Psi_m$, which caused increased ROS and perhaps ATP production. The altered coupling and ROS status resulted in greater depolarization of the plasma membrane, and although still responsive to changes in glucose, the UCP2AKO α -cells showed reduced calcium entry and lower glucagon secretion. **B:** UCP2 expression is normally increased in islets deprived of nutrients. Therefore, lack of UCP2 in α -cells results in impaired glucagon secretion during hypoglycemia. ITT, insulin tolerance test; TCA, tricarboxylic acid.

we showed that β -cell-specific deletion of UCP2 increases insulin secretion in an ROS-dependent manner (18). We now show that the acute addition of exogenous H_2O_2 to control islets increased glucagon secretion under high glucose and reduced glucagon secretion under low glucose conditions, a situation reminiscent of the disordered glucagon secretion from murine UCP2^{-/-} islets (11) and patients with diabetes (1) and human islets exposed to genipin. It is therefore conceivable that UCP2 regulates the levels of ROS in α -cells and, in turn, glucagon secretion. Acute inhibition of UCP2 in Gcg-cre islets with genipin increased endogenous H_2O_2 levels, which correlated with reduced low glucose-mediated glucagon secretion. Similarly, UCP2AKO islets had elevated mitochondrial superoxide levels and signs of chronically altered redox-related signaling pathways (i.e., ROS levels remained high after overnight culture and altered antioxidant gene expression [including reduced *Sod2* expression] was observed), which also correlated with impaired glucagon secretion. Therefore, elevated intracellular ROS can reduce low glucose-mediated glucagon secretion, although the specific ROS species and its signaling targets are unclear.

We propose that α -cell UCP2 expression is normally increased in response to low nutrient levels and limits $\Delta\Psi_m$ hyperpolarization, which, by limiting ATP and/or ROS production, contributes to the cells' ability to secrete glucagon. Therefore, α -cell-specific deletion of UCP2 results in greater depolarization of the plasma membrane and, although still responsive to changes in glucose, reduces calcium entry and lowers overall secretion capacity (Fig. 8A). Deletion of α -cell UCP2 disrupted the hypoglycemic response in mice, implying that increased UCP2 expression/activity, such as that which occurs after fasting, is required to promote glucagon secretion and is protective in this role (Fig. 8B). Conversely, elevated expression or activity of UCP2 may also contribute to the dysregulated glucagon secretion that is observed in the diabetic state.

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E.M.A. researched data, contributed to discussion, wrote the manuscript, and reviewed and edited the manuscript. C.A.R.-D., K.J.P., and A.B.H. researched data, contributed to discussion, and reviewed and edited the manuscript. S.S. researched data and contributed to discussion. H.Y.G. contributed to discussion and reviewed and edited the manuscript. D.K. researched data and contributed to discussion. P.G., P.L.H., and B.B.L. contributed to discussion and

reviewed and edited the manuscript. M.B.W. designed the study, contributed to discussion, and reviewed and edited the manuscript. M.B.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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