Glucagon Is Essential for Adaptive Thermogenesis in Brown Adipose Tissue

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Glucagon, a counterregulatory hormone to insulin, serves as a regulator of glucose homeostasis and acts in response to hypoglycemia. Earlier studies have shown that glucagon administration induces thermogenesis in experimental animal models. However, it is not known whether endogenous glucagon is involved in the regulation of brown adipose tissue (BAT) function. Here we investigated the role of glucagon in cold-induced thermogenesis in male mice deficient in proglucagon-derived peptides (GCGKO mice). Upon exposure to cold, GCGKO mice exhibited a greater decrease in rectal temperature than control mice. The cold exposure-induced increase in oxygen consumption in GCGKO mice was less than that seen in control mice. Moreover, the increase in oxygen consumption after administration of a β3-adrenergic receptor agonist, CL-316,243, was also lesser in GCGKO than in control mice. Expression of thermogenic genes, including the gene encoding uncoupling protein 1 (*Ucp1*), was reduced in the BAT of GCGKO mice under ambient as well as cold conditions. Administration of glucagon restored the expression of *Ucp1* mRNA in the BAT as well as the expression of the fibroblast growth factor 21 gene (*Fgf21*) in the liver. Supplementation with glucagon for 2 weeks resulted in higher plasma Fgf21 levels and improved responses to CL-316,243 in GCGKO mice. These results indicated that endogenous glucagon is essential for adaptive thermogenesis and that it regulates BAT function, most likely by increasing hepatic Fgf21 production. (*Endocrinology* 155: 3484–3492, 2014)

Brown adipose tissue (BAT) is the main site of adaptive thermogenesis in the body, in which energy is dissipated as heat in response to changes in temperature and diet. Recent studies have shown that adult humans have functional BAT that can be activated in response to exposure to cold (1, 2). BAT thermogenesis is directly regulated by the sympathetic nervous system, and the β3-adrenergic receptor expressed in BAT is a major factor in this regulation (3). Upon cold-stimulated activation of BAT via the nervous system, carbohydrates, and lipids are metabolized to produce heat, through the mitochondrial uncoupling protein-1 (UCP1), a protein that uncouples electron transport from ATP production (4). UCP1 synthesis is induced by β-adrenergic stimulation, which also increases the hydrolysis of triglycerides (TGs) in adipose tissues. The fatty acids released in the process activate UCP1 and are oxidized in mitochondria to serve as an energy source for thermogenesis. Together with free fatty acids (FFAs), glucose is an important fuel in BAT, acting as a carbon source for fatty acid synthesis and in the rapid oxidation of fatty acids (5).

Glucagon, which is released from pancreatic α-cells, plays a crucial role in maintaining glucose homeostasis as a counterregulatory hormone to insulin. The main physiological role of glucagon is to stimulate hepatic glucose output, thereby leading to an increase in blood glucose levels. Glucagon also promotes lipolysis in white adipose tissue (WAT) and increases FFA levels in the circulation. In addition to regulating glucose and lipid metabolism, glucagon has been reported to participate in the control of

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Abbreviations: β3AR, β3-adrenergic receptor; BAT, brown adipose tissue; FFA, free fatty acid; FGF21, fibroblast growth factor 21; GCGKO, mice deficient in proglucagon-derived peptides; GLP, glucagon-like polypeptide; TG, triglyceride; UCP1, uncoupling protein-1; WAT, white adipose tissue.

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energy expenditure and thermogenesis. Several animal studies that involved infusion of exogenous glucagon, and in vitro studies of brown adipocytes, have demonstrated that glucagon functions to increase energy expenditure through activation of BAT (6, 7). Furthermore, Seitz et al (8) reported that plasma glucagon levels were significantly elevated after exposure to cold, which implicates glucagon in cold-induced thermogenesis. However, whether endogenous glucagon is involved in cold-induced thermogenesis remains controversial (7).

Fibroblast growth factor 21 (FGF21) is predominantly produced in the liver and is a pleiotropic protein involved in glucose, lipid metabolism, and energy homeostasis (9). Fgf21 expression and secretion are induced in the liver during periods of fasting (10–12) and are stimulated by glucagon signaling (13). Furthermore, a recent study reported that Fgf21 mediates the key metabolic actions of glucagon (14). On the other hand, *Fgf21* mRNA expression is induced in adipose tissues upon exposure to cold stimulation (15, 16); Fgf21 then acts in an autocrine/paracrine manner in adaptive thermogenesis (17). However, it remains unclear whether glucagon is involved in cold-induced Fgf21 expression in BAT.

The present study aimed to clarify the role of endogenous glucagon in cold-induced thermogenesis. For this purpose, we used male mice deficient in proglucagon-derived peptides (GCGKO mice) that are homozygous for a glucagon-green fluorescent protein knock-in allele (18). GCGKO mice lack proglucagon-derived peptides, including glucagon, glucagon-like polypeptide (GLP)-1, and GLP-2. In contrast to mice deficient in the glucagon receptor, which have elevated circulating GLP-1 and reduced blood glucose levels, GCGKO mice are normoglycemic. Therefore, these mice are useful for analyzing the impact of glucagon deficiency under normoglycemic conditions (19, 20). We here also analyzed whether defects observed in the BAT of GCGKO mice could be rescued by supplementation with exogenous glucagon.

Materials and Methods

Animals

All the animal experimental procedures were performed in accordance with the Nagoya University institutional guidelines for animal care, which conform to the National Institutes of Health animal care guidelines. The establishment of GCGKO mice and breeding conditions have been described in detail previously (18). Heterozygous mice were used as a control. The mice used in these experiments had a C57BL/6J genetic background, established through backcrossing for at least 12 generations. All the experiments were carried out using male mice.

Cold exposure

For acute exposure experiments, mice were fasted overnight and were housed individually in cages that had been prechilled at 4°C or were housed at 24°C. Their core body temperature was monitored every 30 minutes using a thermometer (BAT-12; Physitemp Instruments Inc) with a mouse rectal probe (RET-3; Physitemp Instrument Inc).

For metabolic analyses, using the Comprehensive Lab Animal Monitoring System, mice were acclimatized to the monitoring environment overnight. The baseline data were obtained for 1 hour at 24°C. The room temperature was gradually lowered from 24°C to 4°C at a rate of 6°C/h.

CL-316,243 administration

After mice were acclimatized to the monitoring environment for 2 hours, baseline metabolic data were obtained for 2 hours at 24°C. Then CL-316,243 (Sigma-Aldrich Japan) was injected ip at a dose of 1 mg/kg body weight, and after 50 minutes, the data were collected for a period of 2 hours. Foods were deprived during the experimental period, from 4 hours before to 3 hours after the CL-316,243 injection.

Glucagon supplementation experiments

For short-term supplementation, mice were injected sc with glucagon, at a dose of 1 mg/kg body weight (Novo Nordisk Pharma), three times, at 12-hour intervals. Access to food was denied after the second injection. BAT and liver were harvested 2 hours after the last injection.

For long-term supplementation, glucagon (1 mg/kg body weight) was administered once daily for 2 weeks. Animals were exposed to cold or underwent CL-316,243 injection at 24 hours after the last glucagon administration.

Biochemical assays

Blood glucose levels were measured using an Antsense III blood glucose meter (Horiba Ltd). Plasma concentrations of FFAs and TGs were measured enzymatically using the nonesterified fatty acid C and triglyceride E-test kits, respectively (WAKO Pure Chemical). Plasma insulin levels were determined using a mouse insulin ELISA kit (Morinaga-Seikagaku Co Ltd). Plasma Fgf21 concentration was determined by mouse/rat FGF21 ELISA (Biovender Inc). BAT TG contents were measured as described previously (21).

RNA extraction and quantitative PCR

Total RNA was extracted from BAT using the RNAiso Plus reagent (TaKaRa Bio). Total RNA (500 ng) was reverse transcribed using the PrimeScript RT master mix (TaKaRa Bio). Quantitative PCR was performed with the Applied Biosystems StepOne real-time PCR system, using THUNDERBIRD SYBR quantitative PCR mix (Toyobo). The sequences of the primers used and the genes investigated in these analyses are available upon request.

Statistical analysis

Data are presented as means ± SEM. Significance was evaluated using a Student's *t* test or ANOVA, followed by Bonferroni post hoc tests when applicable. A value of $P < .05$ was regarded as statistically significant.

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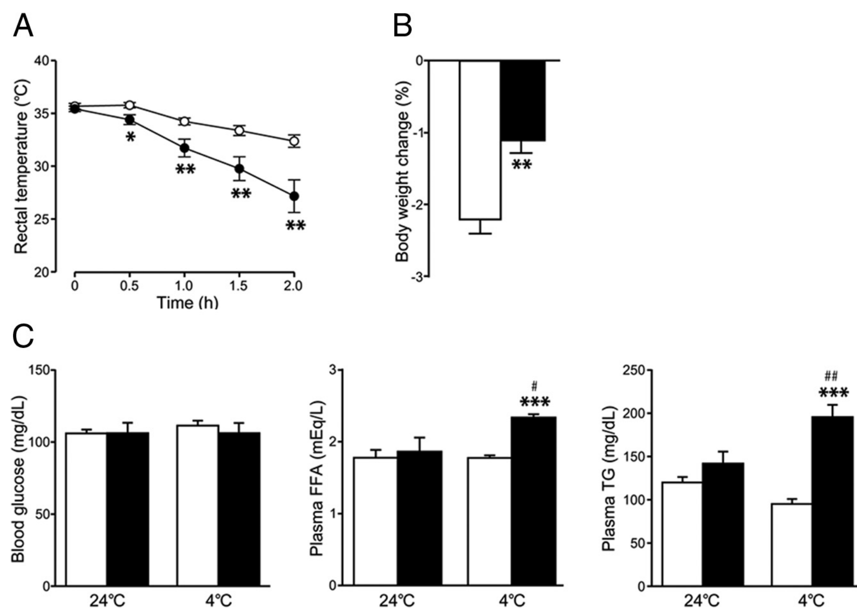

Figure 1. The effect of cold exposure on rectal temperature, body weight, and metabolic parameters. Mice were starved overnight and then housed in cages that had been prechilled at 4°C, or were housed at 24°C, for 2 hours. A, Changes in rectal temperature during cold exposure. Open circles, control mice (n = 11); closed circles, GCGKO mice (n = 10). B, Percentage of body weight change during cold exposure. White bars, control mice (n = 10); black bars, GCGKO mice (n = 8). C, Levels of blood glucose, plasma FFAs, and plasma TGs. White bars, control mice (n = 7); black bars, GCGKO mice (n = 6). Values were expressed as means ± SEM. *, $P < .05$, **, $P < .01$, ***, $P < .001$ vs control; #, $P < .05$, ##, $P < .01$ vs 24°C.

Results

GCGKO mice exhibit cold intolerance

Upon exposure to cold, rectal temperature in GCGKO mice gradually decreased and was significantly lower than that in control mice (Figure 1A). Body weight loss during the cold exposure period was significantly lesser in GCGKO than in control mice (Figure 1B). Plasma levels of FFAs and TGs were higher 120 minutes after cold exposure in GCGKO mice than in control mice, whereas glucose levels were comparable between the two groups (Figure 1C). These findings suggested that the use of lipids for thermogenesis is impaired in GCGKO mice.

Oxygen consumption in both mouse groups gradually increased as the room temperature was lowered. However, GCGKO mice exhibited significantly lower oxygen consumption compared with control mice (Figure 2A). Given that physical activity was comparable between the two groups of mice (Figure 2B), the difference in the oxygen consumption was considered to be due mostly to the differential energy expenditure of their BAT.

Thermogenic response to a β3-adrenergic receptor agonist is impaired in GCGKO mice

To evaluate the thermogenic capacity of the BAT, we investigated the effect of a β3-adrenergic receptor (β3AR) agonist, CL-316,243, on energy expenditure. Both groups showed increased oxygen consumption after CL-316,243 treatment, but the increase in oxygen consumption in GCGKO mice was significantly smaller than that in control mice (Figure 3, A and B). Physical activity was comparable between the two groups of mice (Figure 3C). Treatment with saline did not affect oxygen consumption or physical activity (Supplemental Figure 1). These results indicated that the response to β3-adrenergic stimuli is attenuated in GCGKO mice, similar to the response to cold exposure.

Changes in FFA levels and blood glucose levels in response to β3-adrenergic stimuli were also different between the GCGKO and control mice. Baseline FFA levels in GCGKO mice were significantly higher than those in control mice (Figure 3D); CL-316,243 treatment increased FFA levels in both groups of mice, but at 120 minutes after treatment, FFA levels were significantly lower in GCGKO mice than in control mice (Figure 3D). Blood glucose levels in both groups of mice were decreased at 20 minutes after treatment with CL-316,243 (Figure 3E). Whereas blood glucose levels in the control

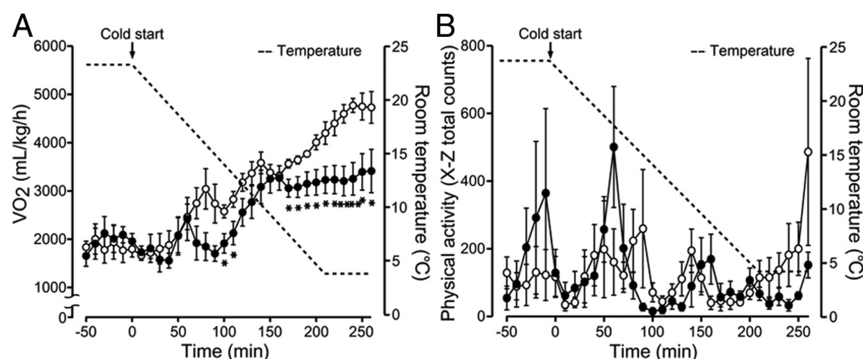

Figure 2. Quantification of energy expenditure. Room temperature was gradually lowered from 24°C to 4°C, and indirect calorimetry data were analyzed using Comprehensive Lab Animal Monitoring System. A, Oxygen consumption (VO_2) during cold exposure. Open circles, control mice (n = 6); closed circles, GCGKO mice (n = 5). B, Physical activity during cold exposure. Open circles, control mice (n = 6); closed circles, GCGKO mice (n = 5) Values were expressed as means ± SEM. *, $P < .05$, **, $P < .01$ vs control.

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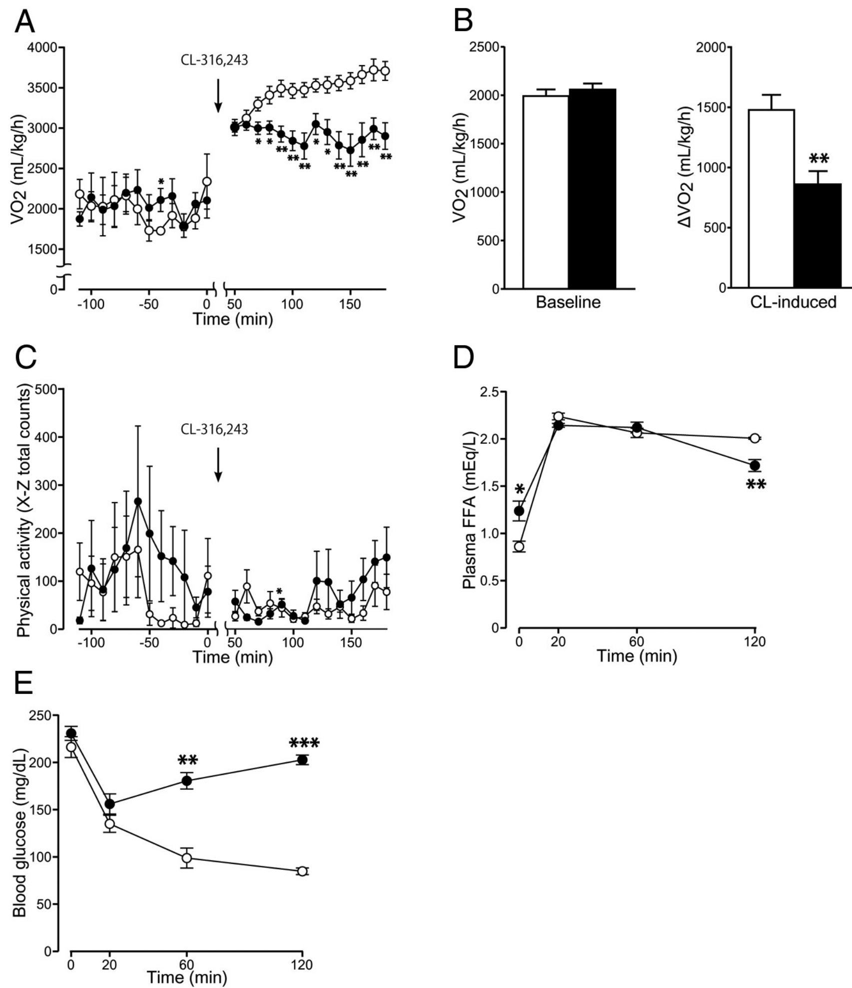

Figure 3. Effect of the β3-AR agonist CL-316,243 on energy expenditure. CL-316,243, at a concentration of 1 mg/kg body weight, was injected ip after starvation for 4 hours. A, Effect of CL-316,243 on oxygen consumption (VO_2). Open circles, control mice (n = 6); closed circles, GCGKO mice (n = 8). B, Baseline oxygen consumption and changes in oxygen consumption due to CL-316,243 treatment. White bars, control mice (n = 6); black bars, GCGKO mice (n = 8). Average oxygen consumption during 2 hours (−120 min to 0 min) before the treatment and that during 2 hours 10 minutes (50 min to 180 min) after the treatment was measured in each animal. C, Effect of CL-316,243 on physical activity. Open circles, control mice (n = 6); closed circles, GCGKO mice (n = 8). D and E, Changes in FFAs (D) and blood glucose (E) after CL-316,243 administration. Open circles, control mice (n = 4); closed circles, GCGKO mice (n = 4). Values were expressed as means ± SEM. *, $P < .05$, **, $P < .01$, ***, $P < .001$ vs. control.

mice remained low at 60 minutes after treatment, those in GCGKO mice had increased and were significantly higher than those in the control mice (Figure 3E).

Cold exposure-induced thermogenic gene expression in BAT is impaired in GCGKO mice

After a 2-hour exposure to cold, BAT weights and BAT TG contents in GCGKO mice were significantly lower than those in control mice (Figure 4, A and B). Hematoxylin and eosin staining showed that the size of lipid droplets was smaller in the BAT of cold-exposed GCGKO than that of cold-exposed control mice (Figure 4C). Because cold exposure induces expression of thermogenic genes, such as *Ucp1* (encoding Ucp1), *Ppargc1a* (encoding Pgc-1α), *Fgf21* (encoding Fgf21), and *Dio2* (encoding D2), in BAT (22), expression levels of these genes were analyzed in both groups of mice. At ambient temperature (24°C), mRNA expression of *Ucp1* was significantly lower in GCGKO than in control mice, and a similar tendency was observed for the expression levels of *Ppargc1a* and *Fgf21* (Figure 4D). Cold exposure induced mRNA expression of *Ucp1*, *Dio2*, and *Ppargc1a* in both groups of mice; however, the increases in expression of these genes were significantly attenuated in the BAT of GCGKO mice (Figure 4D). In addition, *Fgf21* expression in BAT was induced by cold exposure, regardless of the genotype of the mice, suggesting that endogenous glucagon does not contribute to cold-induced *Fgf21* expression.

Cold exposure is known to upregulate the expression of genes involved in glucose metabolism, lipogenesis, and the uptake and catabolism of glucose and fatty acids, thereby activating UCP1 in BAT (5). Therefore, we examined the expression of genes involved in glucose and fatty acid metabolism, viz. those encoding the glucose transporter 4 (*Glut4*), hexokinase 2 (*Hk2*), fatty acid translocase (*Cd36*), and acyl-CoA synthetase long-chain family member 1 (*Acsl1*). At ambient temperature, expression levels of *Cd36* and *Acsl1* were significantly lower in GCGKO than in control mice (Figure 4D). Exposure to cold induced expression of *Hk2* and *Acsl1*, but expression levels in GCGKO mice remained significantly lower than those in control mice (Figure 4D). These results suggested that cold exposure-induced uptake or the use of glucose and fatty acids in GCGKO mice is attenuated compared with that in the control.

Nevertheless, cold exposure-induced reduction in BAT weight and TG content were observed in the GCGKO mice. Therefore, these results suggested that the impaired uptake of fatty acids from the circulation should play some role in the BAT dysfunction in the GCGKO mice.

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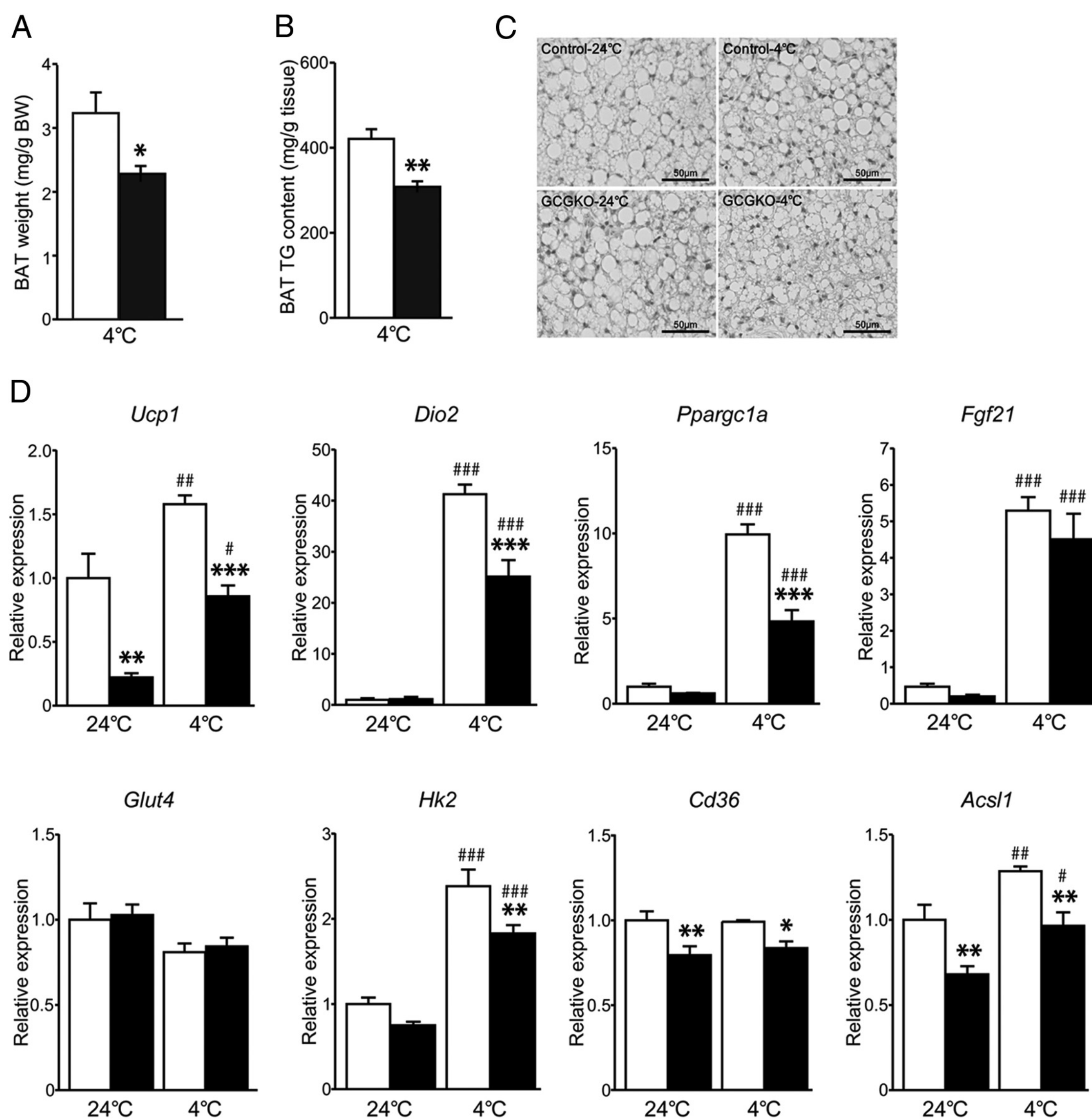

Figure 4. Morphological analysis and thermogenic gene expression in BAT. Mice were housed in cages that had been prechilled at 4°C, or housed at 24°C, for 2 hours. A, BAT weight after cold exposure. White bars, control mice (n = 7); black bars, GCGKO mice (n = 6). B, TG contents in BAT after cold exposure. White bars, control mice (n = 7); black bars, GCGKO mice (n = 5). C, Hematoxylin and eosin staining of BAT. D, mRNA expression of *Ucp1*, *Ppargc1a*, *Dio2*, *Fgf21*, *Glut4*, *Hk2*, *Cd36*, and *Acsl1*. White bars, control mice (n = 5–8); black bars, GCGKO mice (n = 5–6). Values were expressed as means ± SEM. *, $P < .05$, **, $P < .01$, ***, $P < .001$ vs control; #, $P < .05$, ##, $P < .01$, ###, $P < .001$ vs 24°C.

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Supplementation with glucagon improves cold intolerance and BAT function

Gcgr (encoding the glucagon receptor) mRNA expression was higher in the liver than in BAT, and hepatic *Gcgr* mRNA levels were significantly lower in GCGKO than in control mice. On the other hand, these expression levels in BAT did not differ between GCGKO and control mice (Figure 5A).

To establish whether a lack of glucagon is responsible for BAT dysfunction in GCGKO mice, we examined the effect of glucagon administration (3 × 1 mg/kg body weight, every 12 h) on gene expression in BAT and liver tissue.

Glucagon administration significantly increased the mRNA expression of *Ucp1*, *Dio2*, and *Pparg* [encoding peroxisome proliferator activated receptor-γ (*Pparγ*)] in the BAT of GCGKO mice (Figure 5C), whereas the expression of these genes did not change remarkably by exogenous glucagon in control mice (Figure 5B). Expression of *Glut4* and *Hk2* mRNA was increased by glucagon administration in both mice (Figure 5, B and C). These results indicated enhanced sensitivity to glucagon in the BAT of GCGKO mice. The expressions of β3AR mRNA were lower in the GCGKO than in the control mice (Figure 5, B and C), which may partially account for attenuated response to β3-adrenergic stimuli in GCGKO mice.

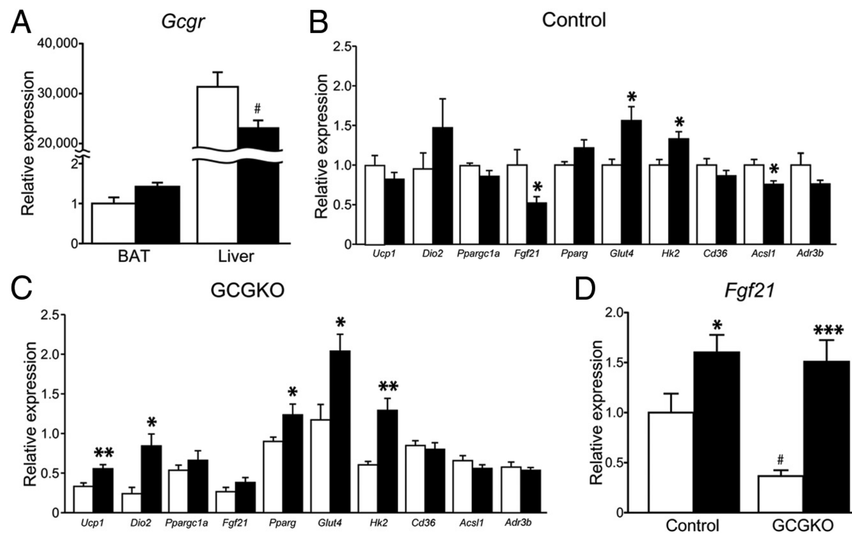

Figure 5. Effect of short-term glucagon treatment on thermogenic gene expression in BAT. Glucagon, at a concentration of 1 mg/kg body weight, was sc injected three times, at 12-hour intervals. A, Expression of *Gcgr* mRNA in BAT and liver. White bars, control mice (n = 8); black bars, GCGKO mice (n = 4–7). B and C, Effect of short-term glucagon treatment on thermogenic gene expression in BAT of control (B) and GCGKO mice (C). White bars, saline-treated mice (n = 5–8); black bars, glucagon-treated mice (n = 6–8). D, Expression of *Fgf21* mRNA in the liver after glucagon administration. White bars, saline-treated mice (n = 7–8); black bars, glucagon-treated mice (n = 7–8). Values were expressed as means ± SEM. *, $P < .05$, **, $P < .01$, ***, $P < .001$ vs saline-treated; #, $P < .05$ vs control.

Glucagon also inhibited *Fgf21* mRNA expression in the BAT of control mice but not in that of GCGKO (Figure 5, B and C). In the liver, *Fgf21* mRNA expression in the GCGKO mice was significantly lower than that in control mice. Glucagon administration increased *Fgf21* mRNA expression to similar levels in the two groups of mice (Figure 5D).

We then tested whether long-term supplementation with glucagon could ameliorate cold intolerance in GCGKO mice. For this purpose, animals that had received daily injections of glucagon for 2 weeks were subjected to cold exposure. As shown in Figure 6A, the body temperature in GCGKO mice that had been injected with saline was decreased to less than 30°C by cold exposure. On the other hand, the decline in body temperature was ameliorated in GCGKO mice supplemented with glucagon, and the decline was comparable with that observed in control mice.

Glucagon supplementation did not affect baseline oxygen consumption under ambient conditions in either GCGKO or control mice. On the other hand, glucagon supplementation significantly enhanced the CL-316,243-induced increase in oxygen consumption in the GCGKO mice but not in the control mice (Figure 6B). At 3 hours after the CL-316,243 injection, blood glucose levels were significantly higher in the GCGKO mice, with or without glucagon supplementation, and glucagon-treated GCGKO mice exhibited lower blood glucose levels compared with those of the saline-treated GCGKO mice. This indicated that their impaired glucose response to CL-316,243 was in part improved by the glucagon supplementation (Figure 6C).

As is shown in Figure 5, the expression of *Hk2* was lower in the GCGKO mice than in the control mice, but glucagon supplementation increased the expression of both *Hk2* and *Glut4* in GCGKO mice. Collectively these results suggested that glucagon regulates glucose use in BAT at the gene expression level, thereby contributing to BAT thermogenesis.

Long-term supplementation with glucagon significantly elevated plasma FGF21 levels upon fasting in GCGKO mice but not in control mice (Figure 6D), and similar data were observed in the *Fgf21* mRNA expression in the liver (Figure 6E). These results indicated that glucagon supplementation, at least partially, ameliorated cold-induced thermogenesis and restored responsiveness to β3-adrenergic stimuli and Fgf21 production in GCGKO mice. On the other hand, lower expression of *Adr3b* mRNA in the BAT in GCGKO mice was not restored by glucagon supplementation (Figure 6F), and this finding suggests that changes in postreceptor signaling is involved in amelioration in thermogenesis.

Discussion

In the present study, we demonstrated that the GCGKO mice, which are deficient in all proglucagon-derived peptides, exhibited cold intolerance and impaired thermogenesis in response to a cold stimulus. Among the proglucagon-derived peptides, glucagon has been postulated to participate in the control of energy expenditure and thermogenesis. Earlier studies have shown that administration of glucagon induces thermogenesis in experimental animals and humans (23). It has also been reported that cold exposure increases plasma glucagon levels (8). These findings suggested that elevated glucagon plays a role in adaptive thermogenesis. In contrast to glucagon, the role of GLP-1 in adaptive thermogenesis is not clear. However, mice deficient in the GLP-1 receptor has been reported to exhibit a normal response to cold exposure and to express

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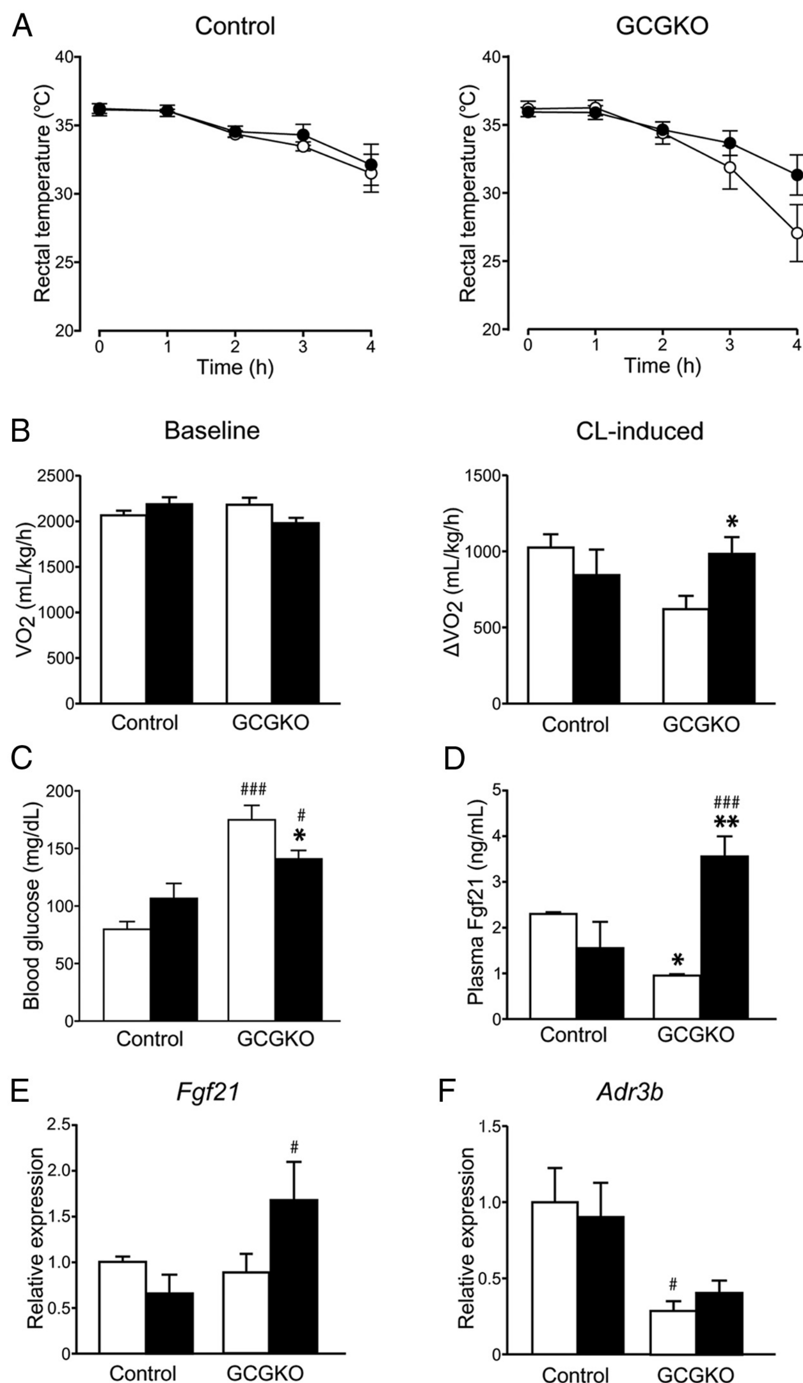

Figure 6. Effect of long-term glucagon supplementation on energy expenditure. Glucagon (1 mg/kg body weight) was administered sc once daily for 2 weeks. Thereafter mice were exposed to cold and CL-316,243 treatment. A, Rectal temperature during cold exposure. Open circles, saline-treated mice (n = 4–5); closed bars, glucagon-treated mice (n = 5). B, Baseline oxygen consumption and changes in oxygen consumption (VO_2) after CL-316,243 treatment. White bars, saline-treated mice (n = 4–5); black bars, glucagon-treated mice (n = 5). C, Levels of blood glucose 3 hours after CL-316,243 treatment in saline-treated and glucagon-treated mice. White bars, saline-treated mice (n = 4–5); black bars, glucagon-treated mice (n = 5). D, Plasma Fgf21 levels on fasting. White bars, saline-treated mice (n = 4); black bars, glucagon-treated mice (n = 4–5). E, Expression of Fgf21 mRNA in the liver after glucagon administration. White bars, saline-treated mice (n = 4–5); black bars, glucagon-treated mice (n = 4). F, Expression of *AdrB3* mRNA in the brown adipose tissue after glucagon administration. White bars, saline-treated mice (n = 4–5); black bars, glucagon-treated mice (n = 4). Values were expressed as means ± SEM. *, $P < .05$, **, $P < .01$ vs saline treated; #, $P < .05$, ###, $P < .001$ vs control.

Ucp1 in BAT at levels comparable with those in control mice (24). The role of other proglucagon-derived peptides, such as GLP-2, in thermogenesis is unknown at present. Therefore, among the proglucagon-derived peptides, glucagon appears to play the most important role in adaptive thermogenesis. In the present study, we demonstrated that cold intolerance in GCGKO mice was partially ameliorated by glucagon supplementation and that glucagon plays physiological roles in regulation of adaptive thermogenesis.

Sympathetic nerve activity has been considered to be the primary physiological signal that activates BAT thermogenesis (4), and β3AR is predominantly expressed in WAT and BAT (25). Activation of the β3AR in WAT enhances lipolysis, and FFAs released from WAT are then consumed as thermogenic fuel in BAT (26). In the present study, we demonstrated that responses to a β3AR agonist, CL-316,243, are attenuated in GCGKO mice and that expression of *Adr3b* mRNA in the lower in GCGKO mice. Intriguingly, replacement of glucagon in GCGKO mice restored the energy expenditure induced by β3-adrenergic stimuli without concomitant increase in *Adr3b* mRNA expression. These findings suggest that glucagon supplementation enhanced responsiveness to β3-adrenergic stimuli through changes in postreceptor intracellular signaling and/or mechanisms independent from β3-adrenergic signaling system. Although βAR signaling is undoubtedly a central regulator of BAT thermogenesis, several other hormones and factors have recently been shown to regulate energy expenditure in adipose tissue (27). Our results demonstrated that a deficiency in glucagon causes cold intolerance and showed that endogenous glucagon is one of the factors involved in the regulation of BAT function.

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UCP1 has been regarded as a key molecule for thermogenesis during cold exposure. Indeed, *Ucp1*-deficient mice have been demonstrated to show a deficient β3-adrenergic-induced activation of thermogenesis and to be cold intolerant (3, 28). We demonstrated that *Ucp1* mRNA expression in BAT is reduced in GCGKO mice and that glucagon administration restored *Ucp1* mRNA expression in the BAT of these mice. These findings indicated that the regulation of Ucp1 expression in BAT in vivo is, directly or indirectly, the major mechanism by which glucagon regulates BAT function. Although in vitro studies have shown that glucagon directly increases energy expenditure via stimulation of oxygen consumption and heat production in brown adipocytes (29, 30), the concentration of glucagon tested in such studies are in the superphysiological range (7). Glucagon released into the blood stream enters the portal vein and first reaches the liver; therefore, the concentration of glucagon perfusing adipose tissues is lower than that in the liver. Furthermore, the expression of *Gcgr* mRNA in BAT was much lower than that in the liver. Taken together, it is likely that glucagon regulates BAT thermogenesis in an indirect manner.

In the present study, the plasma Fgf21 concentration was significantly increased by glucagon supplementation in GCGKO, but not in control mice, and this increased Fgf21 level is most likely due to a release of this factor from the liver. Several lines of evidence have shown that Fgf21 plays an important role in energy homeostasis and that glucagon regulates Fgf21 production. Infusion of Fgf21 has been shown to increase energy expenditure and to elevate core body temperature in mice (31, 32). In addition, in vivo administration of glucagon has been shown to increase circulating FGF21 in healthy or diabetic humans and in rodents with diabetes (13, 33). A recent study showed that the glucagon receptor agonist IUB288 enhanced energy expenditure and induced body weight loss through FGF21 signaling, indicating that Fgf21 mediates the key metabolic actions of glucagon (14). Involvement of endogenous Fgf21 in the regulation of thermogenesis has also been reported (17), and Fgf21 produced in the liver promotes thermogenic activation of neonatal brown fat (34). It has also been reported that exposure to cold induced a marked release of Fgf21 from brown fat, without a concomitant increase in Fgf21 plasma levels, which activated BAT thermogenesis (15, 16). However, the cold-induced increase in *Fgf21* mRNA in BAT seen in this study was comparable between GCGKO and control mice. Therefore, *Fgf21* produced in BAT appears to play a minor role in the thermogenic function of BAT in GCGKO mice.

In conclusion, in this study, we demonstrated that endogenous glucagon is essential for adaptive thermogenesis and that it regulates BAT function, most likely through increasing hepatic Fgf21 production.

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