

Remodeling of Hepatic Metabolism and Hyperaminoacidemia in Mice Deficient in Proglucagon-Derived Peptides

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Glucagon is believed to be one of the most important peptides for upregulating blood glucose levels. However, homozygous glucagon–green fluorescent protein (gfp) knock-in mice (*Gcg^{gfp/gfp}*; GCGKO) are normoglycemic despite the absence of proglucagon-derived peptides, including glucagon. To characterize metabolism in the GCGKO mice, we analyzed gene expression and metabolome in the liver. The expression of genes encoding rate-limiting enzymes for gluconeogenesis was only marginally altered. On the other hand, genes encoding enzymes involved in conversion of amino acids to metabolites available for the tricarboxylic acid cycle and/or gluconeogenesis showed lower expression in the GCGKO liver. The expression of genes involved in the metabolism of fatty acids and nicotinamide was also altered. Concentrations of the metabolites in the GCGKO liver were altered in manners concordant with alteration in the gene expression patterns, and the plasma concentrations of amino acids were elevated in the GCGKO mice. The insulin concentration in serum and phosphorylation of Akt protein kinase in liver were reduced in GCGKO mice. These results indicated that proglucagon-derived peptides should play important roles in regulating various metabolic pathways, especially that of amino acids. Serum insulin concentration is lowered to compensate the impacts of absent proglucagon-derived peptide on glucose metabolism. On the other hand, impacts on other metabolic pathways are only partially compensated by reduced insulin action. *Diabetes* 61:74–84, 2012

Multiple bioactive peptides, including glucagon, glucagon-like peptide 1 (GLP-1), and GLP-2, are produced from proglucagon encoded by the glucagon gene (1,2). Among such peptides, glucagon is believed to play pivotal roles in the regulation of gluconeogenesis and glycogenolysis in the liver, the major target organ of glucagon. Glucagon is produced in pancreatic islet α -cells through cleavage by prohormone convertase 2 (*Pcsk2*) and exerts its action through a glucagon receptor (*Gcgr*). The action of glucagon is mainly mediated by the cAMP-protein kinase A system, which not only activates enzymes through phosphorylation cascades

but also regulates gene expression by modulating the phosphorylation status of transcription factors and transcription coactivators (3).

Animal models defective in *Pcsk2* (*Pcsk2^{-/-}*) and *Gcgr* (*Gcgr^{-/-}*) exhibit blunted glucagon production and action, respectively, and have lower blood glucose levels, thus underscoring the importance of glucagon in glucose homeostasis (4,5). However, because the blunted glucagon action in both *Pcsk2^{-/-}* and *Gcgr^{-/-}* mice induces hyperplasia of α -cells and increases proglucagon production, the serum GLP-1 levels are increased in both types of mice (6,7). Because GLP-1 exerts multiple biological effects, including stimulation of insulin secretion and β -cell proliferation, the possible contribution of increased GLP-1 production to the metabolic phenotypes in these animal models cannot be ignored. Indeed, it has recently been shown that the GLP-1 receptor is indispensable for improvement of glucose homeostasis after blockade of *Gcgr* by glucagon antagonists (8). Therefore, the lower blood glucose levels in *Pcsk2^{-/-}* and *Gcgr^{-/-}* mice are attributable to not only impaired glucagon action but also increased GLP-1 action.

We recently established a mouse model in which part of the glucagon gene (*Gcg*) is replaced by a green fluorescent protein (GFP) cDNA followed by a polyadenylation signal (*Gcg^{gfp/+}*). Homozygous *Gcg^{gfp/gfp}* mice, which lack most, if not all, proglucagon-derived peptides, are born without gross abnormalities, and adult *Gcg^{gfp/gfp}* mice, hereafter referred to as GCGKO mice, display normoglycemia (9). Because GCGKO mice lack both glucagon and GLP-1, this model provides a unique opportunity to analyze the impacts of glucagon deficiency on metabolism without the influence of excess GLP-1. In the current study, we analyzed the hepatic metabolism in GCGKO mice by transcriptome and metabolome profiling to elucidate novel aspects of the physiological roles of proglucagon-derived peptides, especially glucagon.

RESEARCH DESIGN AND METHODS

Animals. The establishment of GCGKO mice and breeding conditions has previously been described in detail (9). All the mice used in the experiments had a genetic background of C57Bl6 J through backcrossing for at least eight generations, except mice used for the microarray analysis, which were offspring of animals backcrossed for three generations. 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.

RNA extraction, microarray analyses, and quantitative PCR. Total RNA was extracted from the livers. Microarray analyses were performed using OpArray Mouse V4.0.1 (Operon Biotechnology, Tokyo, Japan) and a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA). To obtain more rigorous quantitative data, gene expression levels were analyzed by quantitative PCR. The sequences of the primers used for the analyses are available upon request. The details of the procedure have been described previously (10).

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Metabolome analyses. Liver samples were excised from 12–16-week-old male mice, added to five volumes of methanol, and snap frozen in liquid nitrogen. After homogenization, the water-extractable phase was subjected to capillary electrophoresis electrospray ionization mass spectrometry using Agilent CE-TOFMS systems (Agilent Technologies Japan, Tokyo, Japan). The detected peaks were quantified, and each peak was annotated based on the database of Human Metabolome Technologies (Yamagata, Japan), as previously described in detail (11).

Measurement of blood biochemistry and measurement of plasma amino acid concentrations and liver nicotinamide *N*-methyltransferase activity. Serum blood chemistry was analyzed using a Biomajesty JCA-BM2250 automatic analyzer (JEOL, Tokyo, Japan). Heparinized blood samples were obtained from 12-week-old male mice after a 5-h starvation. The plasma concentrations of amino acids were determined by liquid chromatography/mass spectrometry (12). The nicotinamide *N*-methyltransferase (NNMT) activity in the liver homogenates was measured as previously described in detail (13) and expressed as nanomoles of methylnicotinamide produced per hour per gram of liver.

Pyruvate tolerance and glucagon challenge tests. For the pyruvate tolerance test, 1.5 g/kg of sodium pyruvate was administered intraperitoneally to 4-month-old male mice after 18-h fasting. For the glucagon challenge test, 30 μ g/kg of glucagon was administered intraperitoneally to mice fed ad libitum. Tail blood was taken at specified times and the glucose levels were determined using a Medisafe glucose meter (Terumo, Tokyo, Japan).

Indirect calorimetry and measurements of food intake and locomotor activity. Oxygen consumption, CO₂ production, food intake, and locomotor activity were measured using a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). The mice were allowed to acclimatize to the monitoring environment for 24 h, followed by real-time monitoring of the variables under free access to food for 48 h. The mice were also monitored for 24 h without access to food.

Insulin enzyme immunoassay. Serum insulin levels were measured in duplicate using High Sensitivity “PLUS” insulin kit (Morinaga-Seikagaku Co. Ltd., Yokohama, Japan) according to the manufacturer’s instructions.

Antibodies and immunoblot analysis. Antibodies against Akt, phospho-Akt, and β -actin were purchased from Cell Signaling Technology Japan (Tokyo, Japan). The secondary antibodies were purchased from Jackson Immuno-Research (West Grove, PA). The livers were homogenized in ice-cold radioimmunoprecipitation assay buffer (Pierce, Rockford, IL). Aliquots containing 40 μ g of protein were separated on 8% SDS-PAGE gels and transferred onto Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA). Signals were visualized with the Supersignal West Pico Chemiluminescent Substrate (Pierce) and an LAS-1000 lumino-image analyzing system (Fujifilm, Tokyo, Japan).

Statistical analyses. Data are presented as the mean \pm SEM. One-way ANOVA or a two-tailed Student *t* test was applied to all statistical analyses. *P* values of <0.05 were regarded as statistically significant.

RESULTS

Gene expression and metabolome analyses in the GCGKO liver. To evaluate the metabolic status of GCGKO mice, we carried out gene expression and metabolome analyses using capillary electrophoresis mass spectrometry (11) in GCGKO and control livers. The gene expression profiles in the livers of mice starved overnight were analyzed using a two-colored microarray, thus allowing identification of genes differentially expressed between the GCGKO and control livers. The expression levels of such genes were further evaluated by quantitative PCR using cDNA prepared from the livers of GCGKO and control mice under various feeding conditions: fed ad libitum or starved for 5 or 24 h. Although genes encoding rate-limiting enzymes for gluconeogenesis (3) were not identified as differentially expressed genes by the microarray analysis, the expression levels of pyruvate carboxylase (*PYCS*), phosphoenolpyruvate carboxykinase (*PEPCK*), and glucose-6-phosphate phosphatase (*G6PC*) mRNA were also analyzed. As shown in Fig. 1A–C, no significant differences were observed between the control and GCGKO livers for the expression levels of *PYCS*, *PEPCK*, and *G6PC* mRNA. Since there were no differences between the GCGKO and control mice in the concentrations of pyruvate, phosphoenolpyruvate, and glucose-6-phosphate as the substrates

for these enzymes (Fig. 1D), it is considered likely that gluconeogenesis from pyruvates is not severely attenuated in the GCGKO liver.

On the other hand, 10 genes expressed at significantly different levels in the GCGKO livers compared with the control livers were identified through the microarray and quantitative PCR analyses (Fig. 1E). These 10 genes comprised six genes involved in amino acid metabolism, namely soluble glutamate oxaloacetate transaminase (*GOT1*), serine dehydratase (*SDS*), alanine-glyoxylate aminotransferase (*AGXT*), argininosuccinate synthetase 1 (*ASS1*), cystathionine γ -lyase (*CTH*), and ornithine aminotransferase (*OAT*); three involved in fatty acid metabolism, namely fibroblast growth factor 21 (*FGF21*), acetyl-CoA carboxylase α (*ACAC*), and fatty acid-binding protein 5 (*FABP5*); and one involved in nicotinamide metabolism, namely *NNMT*.

Amino acid metabolism. Figure 2A–C depicts the expression levels of the genes encoding enzymes involved in amino acid metabolism, mostly the conversion of amino acids to substrates available for gluconeogenesis or the tricarboxylic acid (TCA) cycle. The interactions of the metabolic pathways mediated by these enzymes are summarized in Fig. 2D. *SDS* converts serine to pyruvate and ammonia (14). *AGXT* is also involved in the metabolism of pyruvate and serine (15). *GOT1* is involved in the malate-aspartate shuttle and thereby in the production of oxaloacetate, which serve as substrates for gluconeogenesis via a *PEPCK*-mediated reaction (16). *SDS*, *AGXT*, and *GOT1* mRNA were expressed at significantly lower levels in the GCGKO livers than in the control livers, especially when the mice were fed ad libitum. The expression levels of *CTH* and tyrosine aminotransferase (*TAT*) mRNA were also significantly lower in the GCGKO livers. *CTH* generates cysteine and α -ketobutyrate from cystathionine, which is synthesized from serine and homocysteine (17), and *TAT* is involved in the conversion of glutamate and α -ketoglutarate (18). *ASS1* and arginase (*ARG*) are involved in the urea cycle, which generates fumarate and urea from *L*-aspartate and carbamoyl phosphate (19,20). *ASS1* produces argininosuccinate from the substrate *L*-citrulline, and the produced argininosuccinate is then converted to *L*-arginine and fumarate. The expression levels of *ASS1* mRNA were significantly decreased in the GCGKO livers. The expression levels of the mRNA encoding *ARG*, which converts arginine to ornithine and ammonia, were also lower in the GCGKO livers than in the control livers, although the difference did not reach statistical significance. The expression levels of the mRNA encoding *OAT*, which is involved in the conversion of α -keto acids and ornithine to *L*-glutamate 5-semialdehyde and *L*-amino acids (21), were also decreased in the GCGKO livers.

The above findings suggest that GCGKO mice have defects in the conversion of amino acids to metabolites available for gluconeogenesis. As shown in Fig. 3A, the relative concentrations of 12 of the 20 amino acids that comprise polypeptides were significantly higher in the GCGKO livers than in the control livers. These findings demonstrate that the altered expression levels of the genes depicted in Fig. 2D result in altered amino acid metabolism. The relative concentrations of arginine, ornithine, *L*-citrulline, and argininosuccinate were also increased in the GCGKO livers, although the difference in the citrulline levels did not reach statistical significance (Fig. 3B). The relative concentration of cystathionine, as the substrate of *CTH*, was also significantly increased in the GCGKO livers (Fig. 3C).

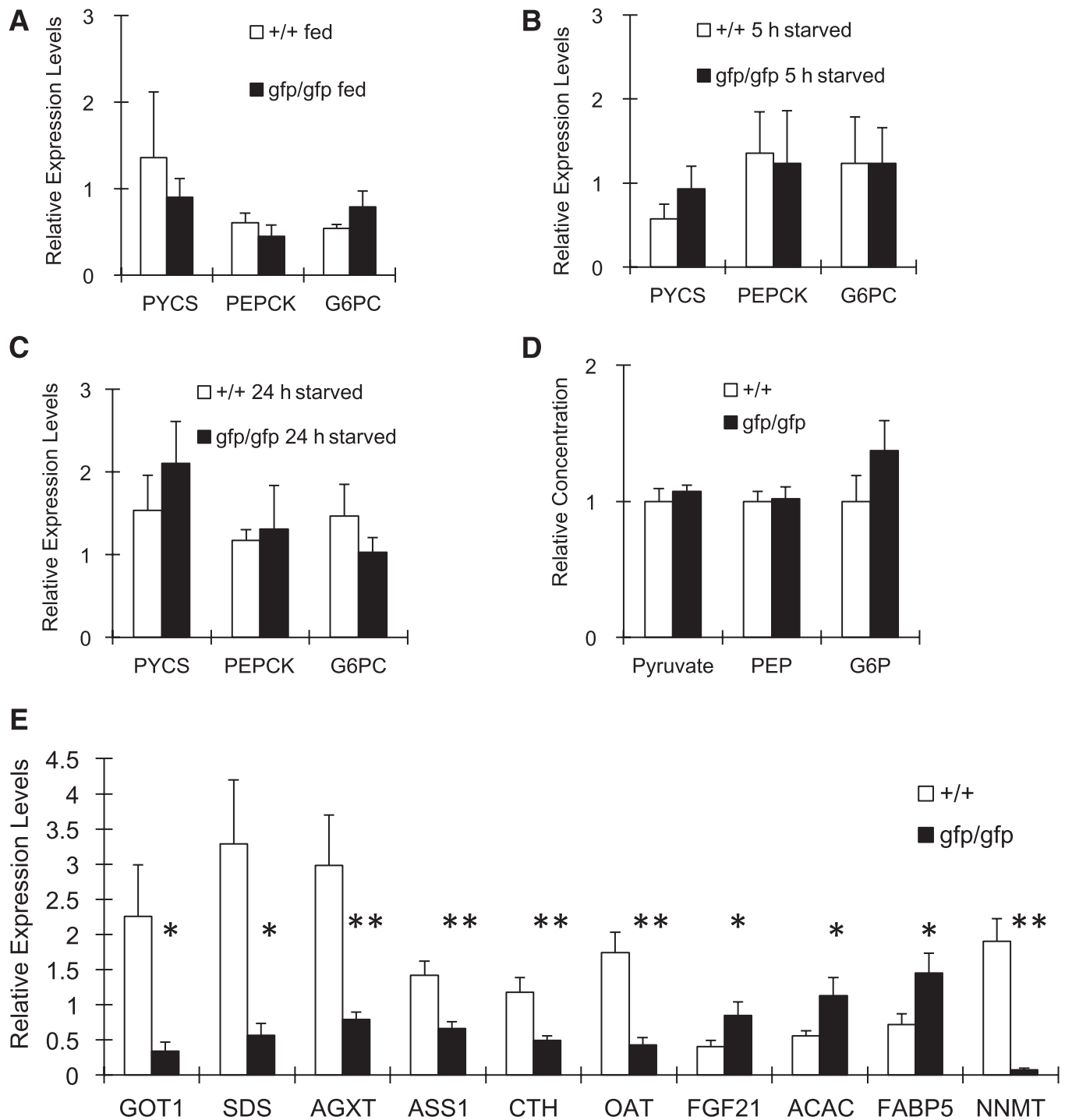


FIG. 1. Expression levels of genes encoding enzymes involved in gluconeogenesis, concentrations of intermediate metabolites for gluconeogenesis, and genes differentially expressed in control and GCGKO livers. Control liver (□); GCGKO liver (■). *A–C*: Expression levels of PYCS, PEPCK, and G6PC mRNA in the liver. The mice were either fed ad libitum (*A*) or starved for 5 (*B*) or 24 h (*C*). The relative expression levels controlled by the expression levels of β -actin mRNA are presented. The data are means \pm SEM ($n = 4–6$). *D*: Relative concentrations of pyruvate, phosphoenolpyruvate, and glucose-6-phosphate in control and GCGKO liver. The data are means \pm SEM ($n = 3$). *E*: The relative expression levels controlled by the expression levels of β -actin mRNA are presented. The data are means \pm SEM ($n = 14–16$). * $P < 0.05$; ** $P < 0.01$.

The plasma amino acid concentrations were subsequently evaluated by high-performance liquid chromatography. It was found that 18 of the 20 amino acids were significantly increased in GCGKO mice, the exceptions being Trp and Phe (Fig. 3*D*). The plasma concentrations of ornithine, citrulline, urea, and cystathionine were also

increased in GCGKO mice (Fig. 3*E*). Collectively, these findings indicate that the altered metabolism in the GCGKO liver results in high concentrations of amino acids in the plasma. Accordingly, it is suggested that peptides derived from proglucagon are required for utilization of amino acids as a source of energy and for gluconeogenesis.

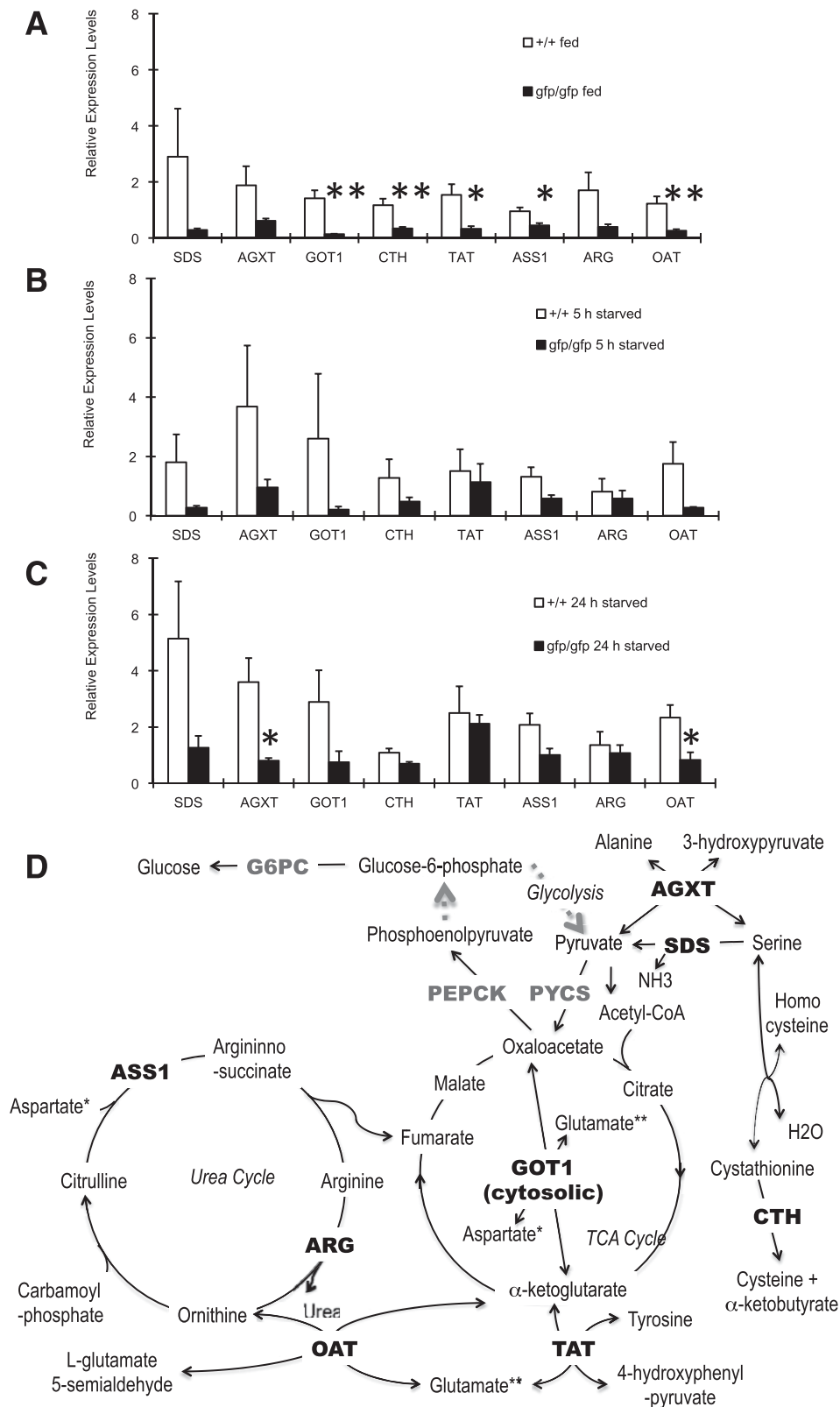


FIG. 2. Expression level of genes encoding enzymes involved in amino acid metabolism and schematic representation of metabolic pathways. **A–C:** Expression levels of mRNA encoding enzymes involved in amino acid metabolism in control (□) and GCGKO (■) liver. The mice were either fed ad libitum (**A**) or starved for 5 (**B**) or 24 h (**C**). The data are means \pm SEM ($n = 4-6$). * $P < 0.05$; ** $P < 0.01$. **D:** Schematic representation of metabolic pathways. The enzymes and metabolites involved in glycolysis, gluconeogenesis, TCA cycle, and urea cycle are summarized. The enzymes analyzed in Fig. 3A–C are shown in bold letters and those analyzed in Fig. 1 are shown in half-tone bold letters. Among the metabolites depicted, aspartate and glutamate are marked with asterisks because they appear twice in this scheme. It should be noted that most of the enzymes analyzed in Fig. 3A–C are involved in the conversion of amino acids to metabolites required for gluconeogenesis via the TCA cycle.

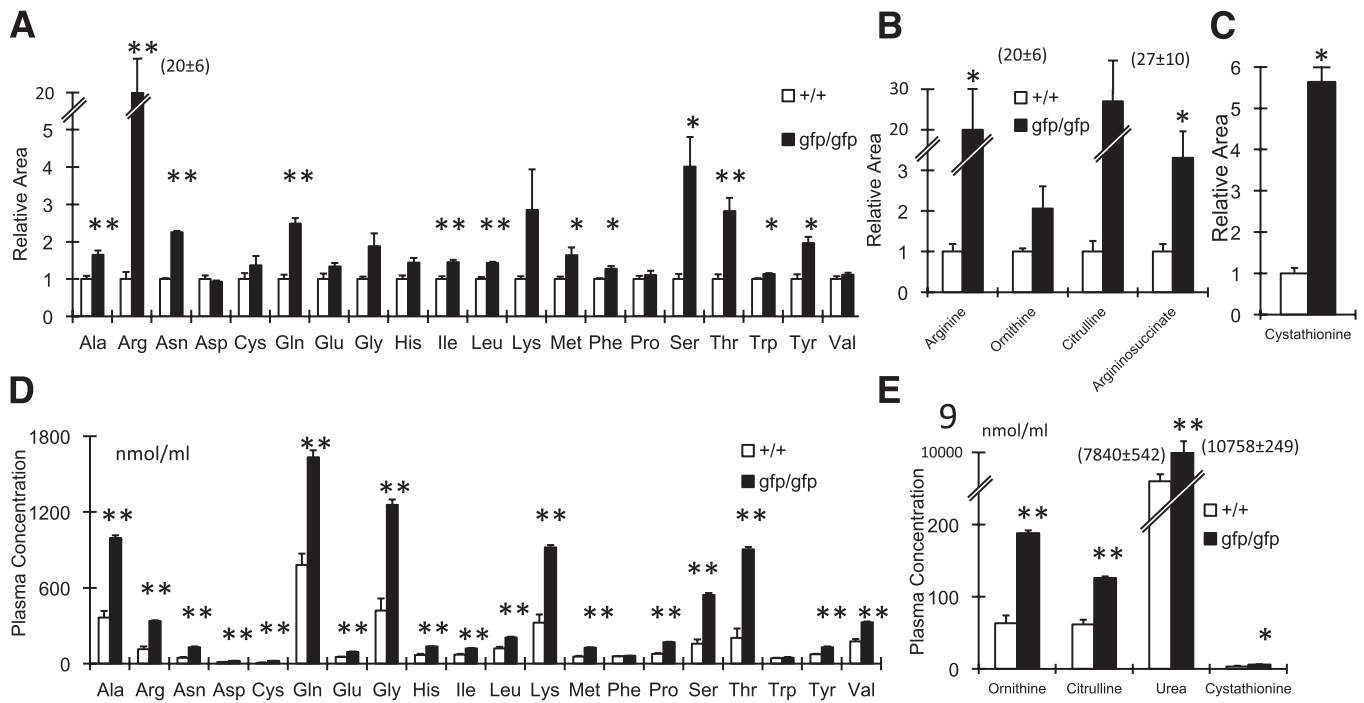


FIG. 3. Relative concentrations of amino acids in liver and plasma. A–C: Relative concentrations of amino acids in control (□) and GCGKO (■) livers. The data are means ± SEM (*n* = 3). **P* < 0.05; ***P* < 0.01. The relative concentrations of the 20 amino acids, urea cycle components, and cystathionine are depicted in A, B, and C, respectively. **D and E:** Plasma concentrations of amino acids and urea. The data are means ± SEM (*n* = 6). **P* < 0.05; ***P* < 0.0001. The concentrations of the 20 amino acids and other variables are depicted in D and E, respectively.

Lipid metabolism. While the expression levels of genes encoding enzymes involved in amino acid metabolism were lower in the GCGKO livers than in the control livers, the expression levels of three genes involved in fatty acid metabolism, *FGF21*, *ACAC*, and *FABP5*, were higher in the GCGKO livers (see Fig. 1E). Figure 4A–C depicts the expression levels of the genes involved in fatty acid metabolism. Similar to the case for the genes involved in amino acid metabolism, the differences between the GCGKO and control livers in the expression of these genes were greatest in the fed state. *ACAC* is involved in the production of malonyl-CoA, thereby serving as the limiting step in lipogenesis (22). On the other hand, *FGF21* is upregulated by starvation and stimulates lipolysis (23,24). *FABP5* is involved in the intracellular transport of fatty acids, which also play an important role in lipolysis (25). These changes in gene expression in the GCGKO livers suggest that both the anabolism and catabolism of fatty acids are increased. Although the serum concentration of triglyceride was decreased, the serum concentrations of HDL, LDL, and total cholesterol were increased in the GCGKO mice (Fig. 4D). Therefore, peptides derived from proglucagon appear to exert both anabolic and catabolic effects on lipid metabolism.

Nicotinamide metabolism. Among the differentially expressed genes, the decreased expression of *NNMT* was most pronounced in the GCGKO livers (Fig. 5A). *NNMT* is involved in the methylation of nitrogen in nicotinamide, and thereby in the excretion and disposal of nicotinamide (26). The enzymatic activity of *NNMT* was significantly lower in the GCGKO livers than in the control livers (Fig. 5B). Figure 5C shows the relative concentrations of nicotinamide and its related metabolites. The mean concentration of *N*-methylnicotinamide in the GCGKO livers was only 17.6

± 3.7% of that in the control livers, whereas the mean concentration of nicotinamide was 75.7 ± 7.7% of that in the control livers (Fig. 5C). Therefore, it is suggested that methylation of nicotinamide is decreased in the GCGKO liver. On the other hand, the concentrations of *NAD*⁺, *NADP*⁺, and *NADPH* were significantly increased, suggesting that the catabolism and excretion of nicotinamide are decreased in GCGKO mice.

Pyruvate and glucagon administration. Upon pyruvate administration, the blood glucose levels were significantly increased in both the GCGKO and control mice, although the increases at 30 and 60 min after the pyruvate injection were significantly greater in the control mice than in the GCGKO mice (Fig. 6A). The differences between the GCGKO and control mice are likely caused by the absence or presence of pyruvate-induced glucagon secretion (27). Nevertheless, since pyruvate also induces gluconeogenesis through mechanisms that are independent of glucagon action, these results indicate that glucagon-independent gluconeogenesis does take place in GCGKO mice.

The effects of glucagon administration were also examined (Fig. 6B). At 15 min after glucagon administration, the blood glucose levels in the GCGKO mice were significantly lower than those in the control mice. The expression levels of the genes depicted in Fig. 1E were not significantly increased by glucagon injection in either the GCGKO or control mice, whereas the expression levels of the *PEPCK* and *G6PC* genes were significantly increased in both the GCGKO and control mice (data not shown). These findings indicate that gluconeogenesis is partially attenuated in GCGKO mice compared with control mice, and suggest that both the lack of proglucagon-derived peptides and the altered gene expression pattern in the liver are involved in the attenuated gluconeogenesis.

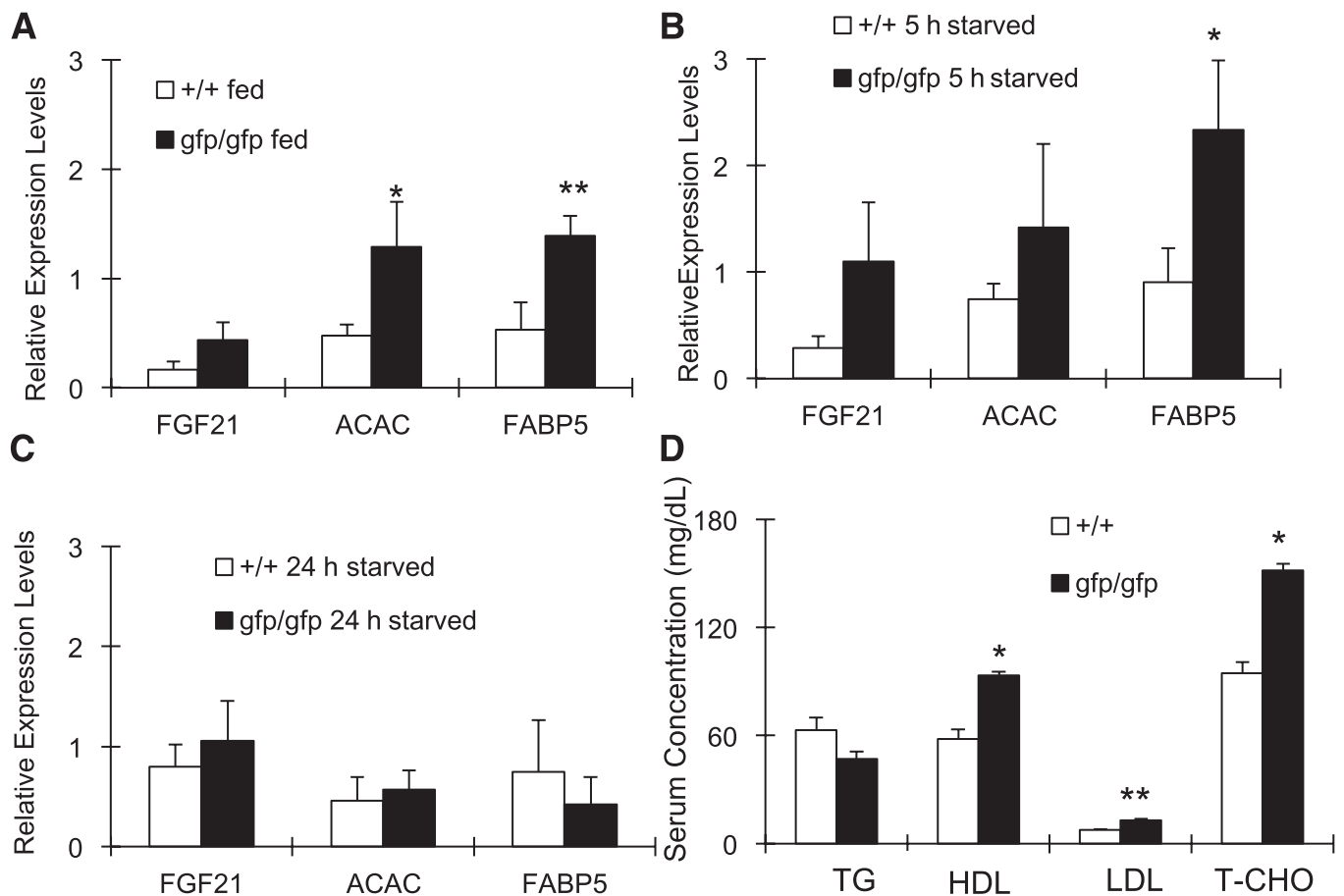


FIG. 4. Expression levels of genes encoding proteins involved in fatty acid metabolism and serum lipid concentrations. *A–C*: Expression levels of FGF21, ACAC, and FABP5 mRNA in control (□) and GCGKO (■) liver. The mice were either fed ad libitum (*A*) or starved for 5 (*B*) or 24 h (*C*). The data are means \pm SEM ($n = 4–6$). * $P < 0.05$; ** $P < 0.01$. *D*: Serum concentrations of triglyceride, HDL, LDL, and total cholesterol. The data are means \pm SEM ($n = 9$). * $P < 0.05$; ** $P < 0.01$.

Energy consumption, food intake, and locomotor activity. Because the combined transcriptome/metabolome analyses suggested impaired utilization of amino acids and altered lipid metabolism, the aspects of whether oxygen consumption and the respiratory exchange ratio (RER) are altered in GCGKO mice were investigated. However, there were no differences in the RERs between the GCGKO and control mice, both with ad libitum feeding and after starvation for 24 h (Fig. 7A). Although the GCGKO mice were heavier than the control mice, no significant difference was observed in the oxygen consumption regardless of the difference in body weight (Fig. 7B) or gross oxygen consumption (data not shown). In addition, there were no significant differences in food intake or locomotor activity (Fig. 7C and D). Collectively, there was no substantial difference in energy consumption between GCGKO and control mice, despite the altered metabolic status of the former.

Plasma insulin levels and Akt phosphorylation in the liver. As blood glucose levels in the GCGKO mice are not significantly different despite lack of glucagon, we evaluated plasma insulin levels in GCGKO mice fed ad libitum. As shown in Fig. 8A, plasma insulin levels were significantly lower in GCGKO than in control mice. We also evaluated phosphorylation of Akt at Ser473 by immunoblot analyses. In concordance with lower serum insulin levels, phosphorylation of Akt was significantly lower in GCGKO

than in control mice (Fig. 8B). These findings suggest that the absence of proglucagon-derived peptides is compensated for, at least in part, by lower serum insulin levels.

DISCUSSION

In the current study, we analyzed the metabolic status of the liver in GCGKO mice, which lack most, if not all, proglucagon-derived peptides, including glucagon and GLP-1. Although the expression of the rate-limiting enzymes for gluconeogenesis at the mRNA level was only marginally altered, the expression of genes involved in the metabolism of amino acids, lipids, and nicotinamides was dysregulated in GCGKO mice.

Amino acids serve as the major source for gluconeogenesis through the removal of amino bases. Oxaloacetate and α -ketoglutarate serve as recipients for amino bases in such reactions and are converted to aspartate and glutamate, respectively. On the other hand, SDS converts serine into pyruvate and ammonia, which is then converted into carbamoyl phosphate. Aspartate, together with carbamoyl phosphate, is converted into urea and fumarate through the urea cycle (see Fig. 2D). Our analyses of the gene expression patterns suggested that the expression of a number of enzymes involved in these procedures was suppressed in the GCGKO liver, and that utilization of amino acids as

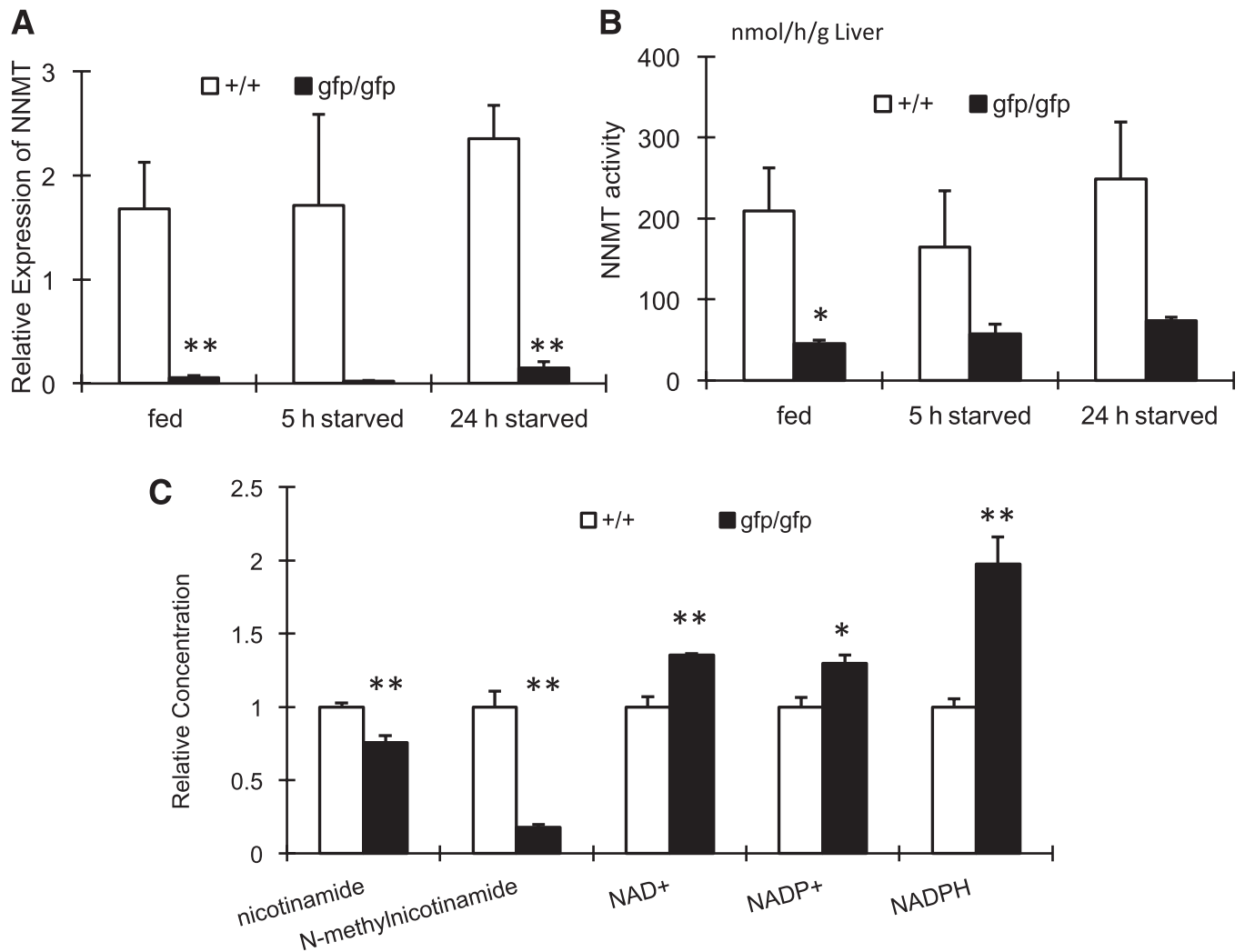


FIG. 5. Gene expression levels and enzymatic activities of NNMT and concentrations of metabolites involved in nicotinamide metabolism. **A:** Expression levels of NNMT mRNA. The data are means \pm SEM ($n = 4-6$). ****** $P < 0.01$. **B:** NNMT activities in the liver. The data are means \pm SEM ($n = 3$). ***** $P < 0.05$. **C:** Relative concentrations of metabolites involved in nicotinamide metabolism. The data are means \pm SEM ($n = 3$). ***** $P < 0.05$; ****** $P < 0.01$.

a source for gluconeogenesis and energy was attenuated. In accordance with our deductions based on the altered gene expression pattern, the concentrations of amino acids were increased in both the liver and plasma of GCGKO mice. Therefore, we concluded that proglucagon-derived peptides play pivotal roles in the regulation of amino acid metabolism. Administration of glucagon or exendin-4, a GLP-1 analog, did not result in increased expression of the genes involved in amino acid metabolism in either the control or GCGKO mice (data not shown). Accordingly, the remodeling of amino acid metabolism is a chronic, rather than an acute, effect of the absence of proglucagon-derived peptides, and mechanisms such as epigenetic regulation (28) are possibly involved in such remodeling.

Taken alone, the current study does not provide answers to the question of which peptide or peptides derived from proglucagon are responsible for the metabolic remodeling in GCGKO mice. However, several reports have supported the pivotal importance of glucagon among the proglucagon-derived peptides in the regulation of amino acid metabolism. Early studies demonstrated that injection of glucagon reduces the plasma amino acid concentrations in healthy humans, whereas suppression of glucagon secretion by injection of somatostatin increases these concentrations

(29,30). It is also known that hypoaminoacidemia is often observed in glucagonoma syndrome (31), and that necrolytic migratory erythema can be successfully treated by infusion of amino acids combined with fatty acids (32,33). Therefore, it is reasonable to consider that the remodeling of amino acid metabolism in GCGKO mice is mainly caused by the absence of glucagon. While the current manuscript was in preparation, it was reported that the concentration of amino acids is increased in the liver of glucagon receptor knockout mice (34). Taken together, among the proglucagon-derived peptides, glucagon appears to play the most important role in the regulation of amino acid metabolism, and the combination of transcriptome and metabolome analyses in the current study has clarified the mechanism for the remodeling of amino acid metabolism evoked by the absence of glucagon.

Although amino acid metabolism was greatly altered in GCGKO mice, their glucose metabolism was only marginally altered. We previously showed that the fasting blood glucose levels in adult GCGKO mice are comparable to those in control mice (9). The mild glycemic phenotype of GCGKO mice is markedly different from those of *Pcsk2*^{-/-} and *Gcgr*^{-/-} mice, which display 20–40% lower blood glucose levels than control mice (4,5). The lower blood

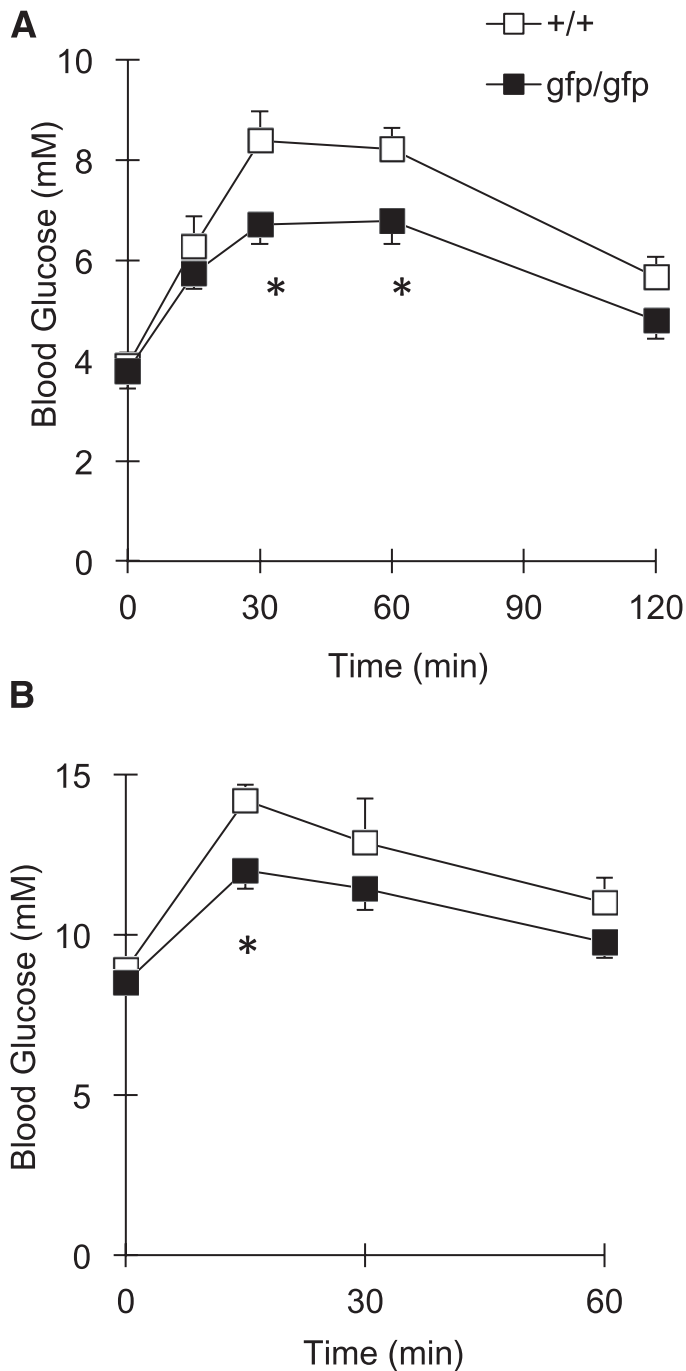


FIG. 6. Pyruvate tolerance and glucagon challenge tests. **A:** For the pyruvate tolerance test, sodium pyruvate was administered at 1.5 g/kg to 4-month-old male mice of the indicated genotypes. The data are means \pm SEM ($n = 6$). * $P < 0.05$. **B:** For the glucagon challenge test, glucagon was administered at 30 mg/kg to 4-month-old male mice of the indicated genotypes. The data are means \pm SEM ($n = 6$). * $P < 0.05$.

glucose levels in the latter models are most likely caused by increased GLP-1 production, which may lower these levels through both insulin-dependent and insulin-independent mechanisms (35). Concordant with the blood glucose levels, the expression levels of genes encoding rate-limiting enzymes for gluconeogenesis did not differ significantly between the GCGKO and control mice (Fig. 1A–D). Analyses of such genes in *Pcsk2*^{-/-} and *Gcgr*^{-/-} mice should further clarify the differential glycemic phenotypes among the animal models deficient in glucagon action.

We previously showed that the serum insulin concentrations in GCGKO mice fed ad libitum are significantly lower than those in control mice (9), and this was reproduced in the current study (Fig. 8A). Recently it has been reported that gluconeogenesis during prolonged fasting is sustained by glucagon-independent mechanisms that involve SIRT1 protein deacetylase and Foxo1 transcription (3,36). For such mechanisms, suppression of insulin signaling due to lower serum insulin levels, but not activation of glucagon signaling, might be required. Therefore, the expression of gluconeogenic genes in the GCGKO liver may be sustained by lower serum insulin levels. In concordance with this assumption, the phosphorylation of Akt was decreased in the GCGKO livers compared with the control livers (Fig. 8B).

The regulatory mechanisms for amino acid metabolism have been far less explored than those for glucose metabolism. On the other hand, the roles of amino acids in the regulation of metabolism, as well as the intracellular signaling pathways involved in such regulation, have recently been investigated. Complexes containing mammalian target of rapamycin respond to diverse extracellular and intracellular cues for promoting anabolic, and inhibiting catabolic, cellular processes. Nutrients, including amino acids, are one of these cues, and amino acids have been shown to regulate the phosphorylation status of p70S6K, S6K1, and eukaryotic initiation factor 4E binding protein through activation of mammalian target of rapamycin complexes 1 and 2 (37–40). Therefore, in GCGKO mice, the increased amino acid concentrations in the liver and plasma, as a consequence of the attenuated utilization for gluconeogenesis and energy production, might promote anabolic processes in GCGKO mice. Although it remains unclear whether protein synthesis is increased in GCGKO mice, it is noteworthy that GCGKO mice display increased body weight, which is proportional to their increased body length (9).

In the current study, we have clarified through combined transcriptome and metabolome analyses that amino acid, but not glucose, metabolism is remodeled in the GCGKO liver. We also found that the expression of genes involved in the metabolism of fatty acids and nicotinamide is altered in the GCGKO liver. However, in the current study, the physiological relevance of the changes in these metabolic pathways could not be explored to the same extent as the changes in amino acid metabolism. It has not been clarified how the metabolism of fatty acids is altered in the GCGKO liver, because the genes involved in fatty acid oxidation and fatty acid synthesis were both expressed at higher levels. Because it has recently been reported that the oxidation of fatty acids is attenuated, whereas their synthesis is increased, in *Gcgr*^{-/-} mice (41), GCGKO mice might harbor similar alterations in fatty acid metabolism. Although no significant differences in the serum concentrations of triglyceride were observed, the serum concentration of cholesterol was increased in GCGKO mice. This increase is most likely caused by reduced recruitment of LDL cholesterol receptor on the surface of hepatocytes (42).

Decreased expression of the *NNMT* gene was the most prominent change in gene expression in the GCGKO liver. Decreased enzymatic activity of NNMT and altered concentrations of related metabolites were also confirmed. Recently, the involvement of nicotinamide in the regulation of various metabolic pathways has been highlighted, because nicotinamide inhibits the enzymatic activity of sirtuins, protein deacetylases/ADP-ribosyltransferases involved in

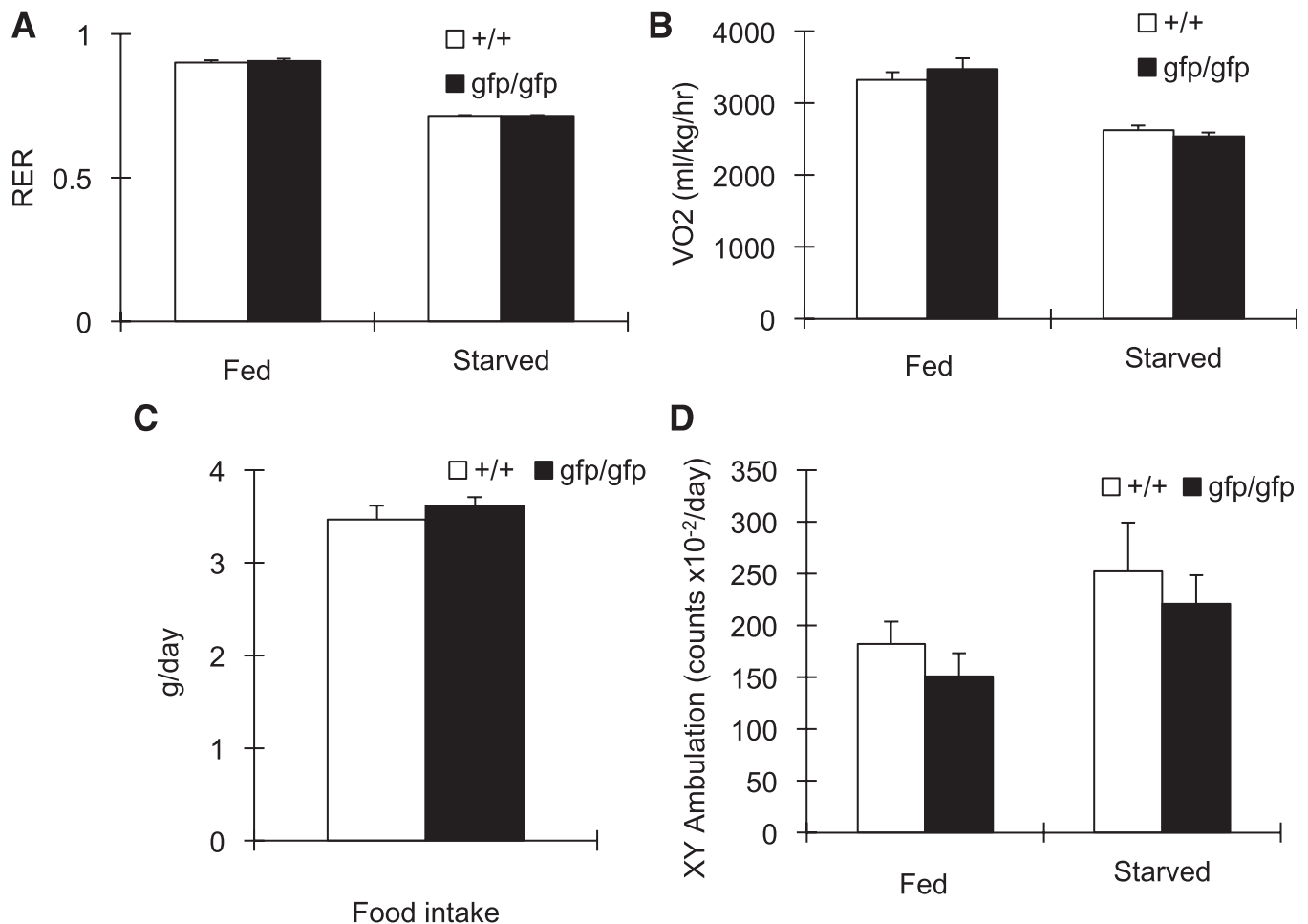


FIG. 7. Indirect calorimetry, food intake, and locomotor activities. *A*: Respiratory exchange ratios of control and GCGKO mice. The respiratory exchange ratios during ad libitum feeding or starvation for 24 h are shown. *B*: Oxygen consumptions of control and GCGKO mice. *C*: Food intake in control and GCGKO mice. *D*: Locomotor activities in control and GCGKO mice. The locomotor activity was monitored by sensor beam lines and horizontal movements in counts ($\times 10^{-2}$) per day are shown. The data are means \pm SEM ($n = 6$) for all variables (*A–D*).

the regulation of many biological processes (43). Therefore, it is interesting to hypothesize that reduced NNMT activity and/or altered nicotinamide metabolism modifies metabolic status by regulating the activity of sirtuins. Accordingly, we analyzed the expression levels of *SIRT1* in the liver, but did not detect any significant difference between GCGKO and control mice in either the mRNA or protein levels (data not shown). Interestingly, suppressed expression of *NNMT* mRNA has been observed in microarray data sets from the liver of mice fed a ketogenic (44) or high fat (45) diet. These findings suggest that *NNMT* expression is regulated by nutritional status. However, the metabolic status of GCGKO mice appears to be different from that of mice fed a ketogenic or high fat diet, because the RER in GCGKO mice did not differ from that in control mice (Fig. 7). Both the regulatory mechanism for *NNMT* mRNA expression and the effects of differential expression of *NNMT* mRNA on metabolic status remain to be elucidated.

In summary, our results have shown a pivotal role for proglucagon-derived peptides in the regulation of not only amino acid metabolism but also fatty acid and nicotinamide metabolism. The results further indicated that the effect of absent glucagon action on glucose metabolism may be compensated by lower serum insulin concentration in GCGKO mice. GCGKO mice should serve as a

unique animal model for analyzing the interactions of various metabolic pathways, including those of glucose, amino acids, lipids, and nicotinamide.

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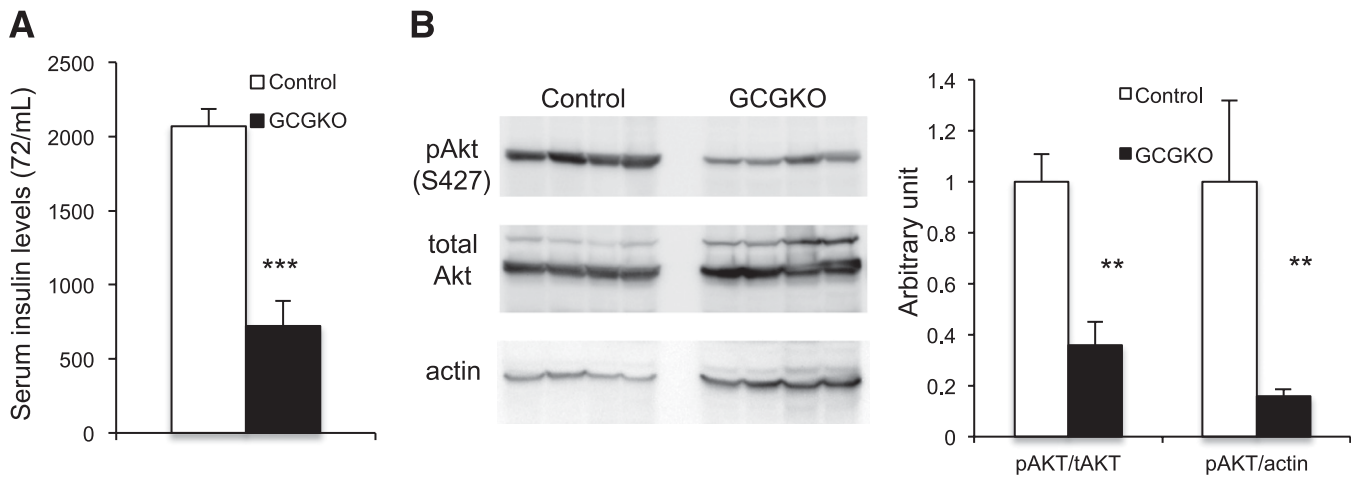


FIG. 8. Serum insulin concentrations and immunoblot analyses of AKT in the liver under ad libitum feeding. **A:** Serum insulin concentrations in the control and GCGKO mice fed ad libitum. The data are presented as means \pm SEM ($n = 6$). *** $P < 0.0001$. **B:** Phosphorylation of Akt. Representative images of immunoblots for phospho-Akt (Thr473), total Akt, and β -actin are shown together with quantitative data. The relative intensities of the phospho-kinase bands were normalized by the band intensities of either β -actin or total Akt and are presented as means \pm SEM. The intensities in the control livers were set as 1 ($n = 4$). ** $P < 0.01$.

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REFERENCES

- Kieffer TJ, Habener JF. The glucagon-like peptides. *Endocr Rev* 1999;20:876–913
- Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. *Gastroenterology* 2007;132:2131–2157
- Altarejos JY, Montminy M. CREB and the CREB co-activators: sensors for hormonal and metabolic signals. *Nat Rev Mol Cell Biol* 2011;12:141–151
- Furuta M, Zhou A, Webb G, et al. Severe defect in proglucagon processing in islet A-cells of prohormone convertase 2 null mice. *J Biol Chem* 2001;276:27197–27202
- Gelling RW, Du XQ, Dichmann DS, et al. Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. *Proc Natl Acad Sci USA* 2003;100:1438–1443
- Conarello SL, Jiang G, Mu J, et al. Glucagon receptor knockout mice are resistant to diet-induced obesity and streptozotocin-mediated beta cell loss and hyperglycaemia. *Diabetologia* 2007;50:142–150
- Gagnon J, Mayne J, Chen A, et al. PCSK2-null mice exhibit delayed intestinal motility, reduced refeeding response and altered plasma levels of several regulatory peptides. *Life Sci* 2011;88:212–217
- Gu W, Winters KA, Motani AS, et al. Glucagon receptor antagonist-mediated improvements in glycemic control are dependent on functional pancreatic GLP-1 receptor. *Am J Physiol Endocrinol Metab* 2010;299:E624–E632
- Hayashi Y, Yamamoto M, Mizoguchi H, et al. Mice deficient for glucagon gene-derived peptides display normoglycemia and hyperplasia of islet alpha-cells but not of intestinal L-cells. *Mol Endocrinol* 2009;23:1990–1999
- Futaki S, Hayashi Y, Yamashita M, et al. Molecular basis of constitutive production of basement membrane components. Gene expression profiles of Engelbreth-Holm-Swarm tumor and F9 embryonal carcinoma cells. *J Biol Chem* 2003;278:50691–50701
- Saito N, Ohashi Y, Soga T, Tomita M. Unveiling cellular biochemical reactions via metabolomics-driven approaches. *Curr Opin Microbiol* 2010;13:358–362
- Shimbo K, Kubo S, Harada Y, et al. Automated precolumn derivatization system for analyzing physiological amino acids by liquid chromatography/mass spectrometry. *Biomed Chromatogr* 2010;24:683–691
- Fukuwatari T, Ohta M, Sugimoto E, Sasaki R, Shibata K. Effects of dietary di(2-ethylhexyl)phthalate, a putative endocrine disrupter, on enzyme activities involved in the metabolism of tryptophan to niacin in rats. *Biochim Biophys Acta* 2004;1672:67–75
- Xue HH, Fujie M, Sakaguchi T, et al. Flux of the L-serine metabolism in rat liver. The predominant contribution of serine dehydratase. *J Biol Chem* 1999;274:16020–16027
- Xue HH, Sakaguchi T, Fujie M, Ogawa H, Ichiyama A. Flux of the L-serine metabolism in rabbit, human, and dog livers. Substantial contributions of both mitochondrial and peroxisomal serine:pyruvate/alanine:glyoxylate aminotransferase. *J Biol Chem* 1999;274:16028–16033
- Kimmich GA, Roussie JA, Randles J. Aspartate aminotransferase isotope exchange reactions: implications for glutamate/glutamine shuttle hypothesis. *Am J Physiol Cell Physiol* 2002;282:C1404–C1413
- Vitvitsky V, Mosharov E, Tritt M, Ataulkhanov F, Banerjee R. Redox regulation of homocysteine-dependent glutathione synthesis. *Redox Rep* 2003;8:57–63
- Hagopian K, Ramsey JJ, Weindruch R. Caloric restriction increases gluconeogenic and transaminase enzyme activities in mouse liver. *Exp Gerontol* 2003;38:267–278
- Jackson MJ, Beaudet AL, O'Brien WE. Mammalian urea cycle enzymes. *Annu Rev Genet* 1986;20:431–464
- Deignan JL, Cederbaum SD, Grody WW. Contrasting features of urea cycle disorders in human patients and knockout mouse models. *Mol Genet Metab* 2008;93:7–14
- Merrill MJ, Pitot HC. Regulation of ornithine aminotransferase by cyclic AMP and glucose in primary cultures of adult rat hepatocytes. *Arch Biochem Biophys* 1985;237:373–385
- Savage DB, Choi CS, Samuel VT, et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J Clin Invest* 2006;116:817–824
- Kharitonov A, Shiyanova TL, Koester A, et al. FGF-21 as a novel metabolic regulator. *J Clin Invest* 2005;115:1627–1635
- Inagaki T, Dutchak P, Zhao G, et al. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* 2007;5:415–425
- Maeda K, Cao H, Kono K, et al. Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metab* 2005;1:107–119
- Yan L, Otterness DM, Craddock TL, Weinshilboum RM. Mouse liver nicotinamide N-methyltransferase: cDNA cloning, expression, and nucleotide sequence polymorphisms. *Biochem Pharmacol* 1997;54:1139–1149
- Ishihara H, Maechler P, Gjinovci A, Herrera PL, Wollheim CB. Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. *Nat Cell Biol* 2003;5:330–335
- Alenghat T, Meyers K, Mullican SE, et al. Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. *Nature* 2008;456:997–1000
- Marliss EB, Aoki TT, Unger RH, Soeldner JS, Cahill GF Jr. Glucagon levels and metabolic effects in fasting man. *J Clin Invest* 1970;49:2256–2270
- Boden G, Rezvani I, Owen OE. Effects of glucagon on plasma amino acids. *J Clin Invest* 1984;73:785–793

31. Chastain MA. The glucagonoma syndrome: a review of its features and discussion of new perspectives. *Am J Med Sci* 2001;321:306–320
32. Bewley AP, Ross JS, Bunker CB, Staughton RC. Successful treatment of a patient with octreotide-resistant necrolytic migratory erythema. *Br J Dermatol* 1996;134:1101–1104
33. Alexander EK, Robinson M, Staniec M, Dluhy RG. Peripheral amino acid and fatty acid infusion for the treatment of necrolytic migratory erythema in the glucagonoma syndrome. *Clin Endocrinol (Oxf)* 2002;57:827–831
34. Lee Y, Wang MY, Du XQ, Charron MJ, Unger RH. Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes* 2011;60:391–397
35. Ayala JE, Bracy DP, James FD, Julien BM, Wasserman DH, Drucker DJ. The glucagon-like peptide-1 receptor regulates endogenous glucose production and muscle glucose uptake independent of its incretin action. *Endocrinology* 2009;150:1155–1164
36. Liu Y, Dentin R, Chen D, et al. A fasting inducible switch modulates gluconeogenesis via activator/coactivator exchange. *Nature* 2008;456:269–273
37. Kimball SR, Siegfried BA, Jefferson LS. Glucagon represses signaling through the mammalian target of rapamycin in rat liver by activating AMP-activated protein kinase. *J Biol Chem* 2004;279:54103–54109
38. Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem* 1998;273:14484–14494
39. Tato I, Bartrons R, Ventura F, Rosa JL. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. *J Biol Chem* 2011;286:6128–6142
40. Dennis MD, Baum JI, Kimball SR, Jefferson LS. Mechanisms involved in the coordinate regulation of mTORC1 by insulin and amino acids. *J Biol Chem* 2011;286:8287–8296
41. Longuet C, Sinclair EM, Maida A, et al. The glucagon receptor is required for the adaptive metabolic response to fasting. *Cell Metab* 2008;8:359–371
42. Rudling M, Angelin B. Stimulation of rat hepatic low density lipoprotein receptors by glucagon. Evidence of a novel regulatory mechanism in vivo. *J Clin Invest* 1993;91:2796–2805
43. Imai S, Guarente L. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. *Trends Pharmacol Sci* 2010;31:212–220
44. Kennedy AR, Pissios P, Otu H, et al. A high-fat, ketogenic diet induces a unique metabolic state in mice. *Am J Physiol Endocrinol Metab* 2007;292:E1724–E1739
45. Recinos A 3rd, Carr BK, Bartos DB, et al. Liver gene expression associated with diet and lesion development in atherosclerosis-prone mice: induction of components of alternative complement pathway. *Physiol Genomics* 2004;19:131–142