CITRIN/MITOCHONDRIAL GLYCEROL 3-PHOSPHATE DEHYDROGENASE DOUBLE-KNOCKOUT MICE RECAPITULATE FEATURES OF HUMAN CITRIN DEFICIENCY.

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Running title: Citrin/mGPD double KO mice as a model of human citrin deficiency

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Citrin is the liver-type mitochondrial aspartate-glutamate carrier that participates in urea, protein and nucleotide biosynthetic pathways by supplying aspartate from mitochondria to the cytosol. Citrin also plays a role in transporting cytosolic NADH reducing equivalents into mitochondria as a component of the malate-aspartate shuttle.

In humans, loss-of-function mutations in the SLC25A13 gene encoding citrin cause both adult-onset type II citrullinemia and neonatal intrahepatic cholestasis; collectively referred to as human citrin deficiency.

Citrin-knockout mice fail to display features of human citrin deficiency however, despite measurable metabolic deficits. Based on the hypothesis that an enhanced glycerol phosphate shuttle activity may be compensating for the loss of citrin function in the mouse, we have generated mice with a combined disruption of the genes for citrin and mitochondrial glycerol 3-phosphate dehydrogenase. The resulting double-knockout mice demonstrated citrullinemia, hyperammononia that was further elevated by oral sucrose
administration, hypoglycemia, and a fatty liver; all features of human citrin deficiency. An increased hepatic lactate/pyruvate ratio in the double-knockout mice compared to controls was also further elevated by the oral sucrose administration, suggesting that an altered cytosolic NADH/NAD⁺ ratio is closely associated with the hyperammonemia observed. Microarray analyses identified over 100 genes that were differentially expressed in the double-knockout mice compared to wild-type controls, revealing genes potentially involved in compensatory or down-stream effects of the combined mutations. Together, our data indicate that the more severe phenotype present in the citrin/mitochondrial glycerol 3-phosphate dehydrogenase double-knockout mice represents a more accurate model of human citrin deficiency than citrin-knockout mice.

Human citrin deficiency is a newly-established disease entity (1,2) that encompasses both adult-onset type II citrullinemia (CTLN2; Online Mendelian Inheritance in Man [OMIM] no. 603471) and neonatal intrahepatic cholestasis (NICCD; OMIM no. 605814), and results from mutations in the SLC25A13 gene that encodes citrin (3). Citrin and the closely related protein aralar (encoded by SLC25A12) (4) are isoforms of the aspartate (Asp)-glutamate (Glu) carrier (AGC) found within the inner mitochondrial membrane and are responsible for the electronegative exchange of Asp for Glu and a H⁺ ion (5). Citrin is predominantly found in liver, kidney, heart and small intestine, while aralar is found in brain, skeletal muscle, kidney and heart (3,6,7). Based on their partially overlapping expression patterns, citrin can be thought of as the liver-type, while aralar the brain- and muscle-type, AGC. Both proteins have a characteristic bipartite structure; the C-terminal half of each protein contains the canonical mitochondrial solute carrier structure while their N-terminal half contains EF-hand motifs that have been shown to bind calcium (8,9), stimulating their transport activities (5). The function of the AGC is to provide substrate for the synthesis of proteins, nucleotides and urea, in addition to participating in gluconeogenesis from lactate (Lac) and transporting cytosolic NADH reducing equivalents into mitochondria as part of the malate-Asp (MA) shuttle.

Citrullinemia is caused by a deficiency of the urea cycle enzyme argininosuccinate synthetase (ASS), which catalyzes the ligation of citrulline (Cit) and Asp to form argininosuccinate at the expense of ATP utilization. Saheki et al. (10) originally described three clinical forms of citrullinemia based on ASS enzyme abnormalities; these forms have subsequently been re-classified into classical citrullinemia (CTLN1; OMIM no. 215700) caused by mutations in the ASS gene (11,12) and CTLN2 caused by mutations in SLC25A13 (3). CTLN2 is characterized by a liver-specific decrease in ASS protein (13) without any detectable abnormalities in the ASS gene or hepatic ASS mRNA levels (14,15). The causal link between citrin deficiency and the hepatic loss of ASS in CTLN2 patients still remains to be clarified (16). Patients with CTLN2 suffer from recurring neuropsychiatric symptoms associated with hyperammonemia, including disorientation, delirium, seizures, and coma that can lead to death from brain edema (13). Laboratory findings of CTLN2 patients
show moderately elevated plasma Cit and arginine (Arg) levels, an elevated plasma threonine to serine (Thr/Ser) ratio, a decreased Fischer ratio (branched-chain amino acids to aromatic amino acid ratio; BCAA/AAA) (17), an elevated serum level of pancreatic secretory trypsin inhibitor (PSTI) (18), and fatty liver (19). The elevated PSTI in CTLN2 patients results from its up-regulated transcriptional expression in the liver (20). Finally, CTLN2 patients also have an increased incidence of hepatocellular carcinoma, pancreatitis and hyperlipidemia (1,2,8,21-30).

Clinically, one of the most distinct features of CTLN2 are reports of patients having a peculiar fondness for foods high in protein and fat such as beans and nuts, and a dislike of foods high in carbohydrates such as rice and sweets (1,2,13,27). Patients with CTLN1 or other urea-cycle enzyme deficiencies typically are given restricted protein diets to minimize episodes of hyperammonemia resulting from protein degradation. In contrast, several CTLN2 patients have developed severe hyperammonemia or deterioration following an intravenous infusion of a high glucose solution (31,32) or an administration of glycerol and fructose for the treatment of brain edema (33). Furthermore, we have observed a citrin deficiency patient who exhibited an increased blood ammonia level following a typical hospital meal, but showed no increase following a meal rich in protein and fat (our unpublished data). Yajima et al. (34) have reported previously that the blood ammonia levels of CTLN2 patients demonstrate diurnal fluctuations; the levels are usually higher in the evening following a meal and low prior to a meal. Together these observations suggest that CTLN2 patients may present with hyperammonemia as a result of dietary intake of carbohydrates, and not protein, following a meal.

Identification of mutations in SLC25A13 in CTLN2 patients led to the discovery that a type of neonatal hepatitis was also caused by the same mutations (35-37). Since the neonatal symptoms were markedly different from those of adult CTLN2 patients, we named the neonatal presentation NICCD (neonatal intrahepatic cholestasis caused by citrin deficiency) (1,38). Patients with NICCD show multiple metabolic abnormalities, including variable aminoacidemias (Cit, Thr, methionine, tyrosine, and Arg) accompanied by an increased Thr/Ser ratio, galactosemia, hypoproteinemia, hypoglycemia, cholestasis, and fatty liver (2). Human citrin deficiency therefore results in NICCD during the first few months of life, with symptoms usually self-resolving by the first year in most cases. Following an apparently healthy period that can last from one to several decades, some patients with human citrin deficiency go on to develop severe CTLN2 (1,36,39). There have been a few patients with NICCD that have required liver transplantation due to severe, prolonged symptoms (40,41), and patients presenting with CTLN2 typically continue to worsen unless also treated by liver transplantation (1,8,23,26,39).

To fully understand the pathophysiology of human citrin deficiency and to aid in the development of novel therapeutics effective in treating NICCD and CTLN2 patients, it is critical to have an accurate animal model of human citrin deficiency. We have previously created a citrin (Ctn)-knockout (KO) mouse (42) by targeted disruption of the Slec25a13 gene.
Although the resulting Ctn−/− mice lacked Slc25a13 mRNA and citrin protein, and demonstrated markedly reduced mitochondrial Asp transport and MA shuttle activity in vitro, there was no apparent phenotype in vivo. Nitrogen-loading experiments produced only minor changes in hepatic ammonia and amino acid levels in vivo. The mice however, did reveal deficits in ureogenesis from ammonia, gluconeogenesis from Lac and an increase in the Lac to pyruvate (Pyr) (L/P) ratio during liver perfusion experiments. Mouse liver contains high mitochondrial glycerol 3-phosphate dehydrogenase (mGPD) activity (43, our unpublished data), a component of the glycerol phosphate (GP) shuttle that can similarly transport NADH reducing equivalents into mitochondria, while human liver is known to contain much lower activity (44, our unpublished data). These considerations led us to hypothesize that the higher GP shuttle activity in the liver of the Ctn−/− mice may be sufficient to compensate for any decreases in MA shuttle activity resulting from the loss of citrin function.

To test this hypothesis, we crossed mGPD-KO mice to Ctn-KO mice. The resultant Ctn/mGPD double-KO (Ctn+/−, mGPD−/−) mice revealed hyperammonemia under fed conditions, citrullinemia and hypoglycemia under fed and fasted conditions, and an altered Thr/Ser ratio and fatty liver under fasted conditions. Moreover, the hyperammonemia was enhanced by the administration of sucrose, with the rise in ammonia being closely associated with an increased L/P ratio. Finally, microarray gene expression analyses using liver RNA identified more than 100 genes that were differentially expressed in the double-KO mice compared to wild-type controls, only a portion of which were also differentially expressed in either of the Ctn-KO or mGPD-KO mice. These results support our contention that compensatory mechanisms in mouse are responsible for the observed differences in citrin deficiency between humans and mice, and that the more severe phenotype present in the double-KO mice represent a more accurate model of human citrin deficiency.

**EXPERIMENTAL PROCEDURES**

**Animals** - Mice homozygous for the targeted disruption of the Slc25a13 gene that encodes citrin (Ctn+/− or Ctn-KO) and for the mGPD gene that encodes mGPD (mGPD+/− or mGPD-KO) were generated as described previously (42,45). Both KO mutations were made congenic on the C57BL/6J genetic background by backcrossing for at least nine generations. The two KO strains were intercrossed to generate mice heterozygous for both mutations (Ctn+/−, mGPD+/−), which in turn were intercrossed to obtain mice that were heterozygous for citrin and homozygous for mGPD (Ctn+/−, mGPD−/−). These mice were then intercrossed to generate single mGPD-KO (Ctn+/+, mGPD−/−) and double-KO (Ctn−/−, mGPD−/−) littermates. Single Ctn-KO (Ctn+/−, mGPD+/−) and C57BL/6J wild-type mice were also generate as littermates by intercrossing mice heterozygous for the Ctn-KO mutation. Genotyping was performed on DNA extracted from ear punch using procedures specific for each of the targeted disruptions made in the Ctn-KO and mGPD-KO mice as described previously (42,45).

**Animal treatment** - All mice were maintained at constant temperature (22 ± 1 °C) on a 12 hour
light/12 hour dark cycle (7 a.m. to 7 p.m.) with free access to water and CE2 chow (24.9% protein, 4.6% fat, and 51.4% carbohydrate providing 346.8kcal/100g; CLEA Japan, Tokyo, Japan). Mice used for experiments were analyzed between 80 and 140 days of age. In each experiment, both genders were analyzed and the results pooled within each genotype if no differences between genders were observed. A sucrose solution (50% w/v of water; 10g/kg body weight) was administered *per os* using a gastric tube to fed mice between 9-10 a.m. For fasting experiments, food was withdrawn at 5 p.m. on the day prior to performed experiments. This study was approved by the Ethical Committee for Animal Experimentation at Kagoshima University.

**Determination of plasma parameters** - Blood was drawn from the mice via cardiac puncture while under general anesthesia (intraperitoneal injection of pentobarbital, 50 mg/kg body weight) between 10-12 a.m. Plasma glucose and blood ammonia concentrations were assayed using Glucose CII-test Wako and Ammonia-test Wako kits (Wako Pure Chemical Industries, Osaka, Japan), respectively, and plasma insulin concentration, using Mouse Insulin ELISA (OPD) (AKRIN-011, Shibayagi, Gunma, Japan). Plasma amino acid concentrations were determined with a JLC-500 model amino acid analyzer (JEOL, Tokyo, Japan) after deproteinization with 3% sulfosalicylic acid. Plasma triglycerides (TG; TG-II Kainos; Kainos Laboratories, Inc., Tokyo, Japan), free fatty acids (FFA; Determiner-NEFA; Kyowa Medix Co. Ltd., Tokyo, Japan), total cholesterol (Cholesterol E-test Wako), glycerol (GLY 105; Randox Laboratories, Antrim, QY, United Kingdom), ketone bodies (Ketone Test Sanwa; Sanwa Kagaku Kenkyusho, Nagoya, Japan), bile acid (Bile Acid Test Wako), Asp aminotransferase (AST; TA-LN Kainos), alanine (Ala) aminotransferase (ALT; TA-LN Kainos), and alkaline phosphatase (ALP; Alkaline phosphatase K-test Wako) were determined with commercially-available kits as indicated, respectively.

**Determination of urea-cycle enzyme activities** - Liver extracts were prepared as described previously (46). The urea-cycle enzyme activities were determined using the methods of Schimke (47) for carbamoylphosphate synthetase (CPS), Pierson et al. (48) for ornithine carbamoyltransferase (OCT), Su et al. (49) for ASS and argininosuccinate lyase (ASL), and Ruegg et al. (50) for arginase.

**Quantification of liver TG and Asp** - Total lipid was extracted from the liver with 20 volumes of chloroform-methanol solution (v/v, 1:2) as described previously (51). TG was assayed using a commercially-available kit (TG-EN Kainos). For the determination of hepatic Asp, a portion of liver was excised following blood withdrawal, quickly freeze-clamped and homogenized with 20 times volume of 2% (w/v) trichloroacetic acid after pulverization under liquid nitrogen. The resultant extract was diluted with an eluent and subjected to reversed-phase column chromatography with an ion pair reagent (LC-10Avp series HPLC system; Shimadzu, Kyoto, Japan). Asp was detected using a mass spectrometer with an electrospray source operated in positive ionization mode (single quadrupole mass spectrometer AQA, Thermo Fisher Scientific Inc. MA, USA), and observed at m/z 133.1 (protonated molecular ion).
**Determination of hepatic lactate and pyruvate concentrations** – Following cervical dislocation, a portion of liver was quickly freeze-clamped, pulverized under liquid nitrogen and homogenized with 3% perchloric acid. Lac and Pyr in the neutralized extract were determined using the Determiner LA kit (Kyowa Medix Co. Ltd., Tokyo, Japan) and by the method of Lamprecht and Heinz (52), respectively.

**Microarray analysis** - Mouse livers were quickly removed, freeze-clamped, weighted, and placed in TRIZOL reagent (Invitrogen, Carlsbad, CA) for the isolation of total RNA according to the manufacturer’s instructions. The quality and quantity of isolated RNA was assessed on a Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) prior to its use in two-color microarray experiments. The RNA from four wild-type mice was equally pooled and used as a common reference for comparisons with four individual samples each from mGPD-KO, Ctn-KO and double-KO mice. For cDNA and the subsequent fluorescent cRNA syntheses, 150 ng of total RNA was used with the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Cyanine 5-dCTP (Cy5) and cyanine 3-dCTP (Cy3) nucleotides (PerkinElmer, Boston, MA, USA) were used to label all the test and reference cRNA probes, respectively. Labeled cRNA probes were purified using the RNeasy kit (Qiagen, Valencia, CA), and added (750 ng each) to the hybridization mixture from the In Situ Hybridization Kit Plus (Agilent). Following fragmentation, each mixture was hybridized for 17 hours at 60°C to the Whole Mouse Genome Oligo Microarray (44K-format; Agilent), washed twice using SSC buffers with Triton X-102 according to the manufacturer’s recommendations and scanned for Cy3 and Cy5 fluorescence using an Agilent DNA Microarray Scanner (model G2565BA). Data were extracted from the scanned images with Feature Extraction Software ver7.5 (Agilent Technologies) using the default settings. Lowess (locally weighted linear regression curve fit)-normalized Cy5/Cy3 ratios were used to perform statistical tests (with a false-discovery rate < 0.05) to identify genes demonstrating significantly altered expression levels (thresholds corresponding to a 2-fold change) using the GeneSpring Software (Agilent).

**Real-time PCR for quantification of gene expression** - Total RNA was isolated from mouse livers using the method of Chomczynski and Sacchi (53). First-strand cDNA synthesis was performed using 10 μg of total RNA and oligo-(dT)12-18 priming following the manufacturer’s instructions (First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed using SYBR-green chemistry on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s recommendations. The cycle threshold number (CT) at which amplification entered the exponential phase was determined for each gene under investigation and used as an indicator of the relative amount of initial target RNA in each sample. Analysis of gene expression levels was performed using the comparative CT method, determining target gene expression relative to an internal control and to an individual control sample. The glyceraldehyde 3-phosphate dehydrogenase (Gapdh) gene was used as an internal control for all samples.
Gapdh expression did not change among the four genotypes; no statistical difference was found in CT values for wild-type (19.7 ± 0.3), Ctrn-KO (20.9 ± 2.2), mGPD-KO (20.2 ± 0.8), and Ctrn/mGPD double-KO (20.9 ± 0.6) mice (each n = 3). All primers used for real-time PCR are shown in Table 1.

Statistical Analysis - All data are presented as means ± SD. Differences among multiple group means were evaluated by one-way analysis of variance (ANOVA) following by the Tukey-Kramer test. Differences between only two group means were tested using a Student’s t-test. Differences between expected and observed frequencies were tested using a chi-square test. JMP (SAS Institute Inc., Cary, NC, USA) and Excel software (2002, Microsoft Corporation, USA) were used for all statistical analyses. A p-value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Decreased survival and growth retardation of Ctrn/mGPD double-KO mice - Based on our breeding scheme of intercrossing mice heterozygous for the Ctrn-KO mutation and homozygous for the mGPD-KO mutation (Ctrn+/+, mGPD-/- X Ctrn+/+, mGPD-/-), we expected a 1:2:1 ratio of mGPD-KO mice (Ctrn+/-, mGPD-/-; n = 146), mGPD-KO mice heterozygous for the Ctrn-KO mutation (Ctrn+/+, mGPD-/-; n = 285) and Ctrn/mGPD double-KO mice (Ctrn-/-, mGPD-/-; n = 106) at the time of genotyping. All mice were genotyped between 20 and 40 days of age, and by this age, there was a 30% loss of Ctrn/mGPD double-KO mice (χ² = 7.99, p < 0.02). There was no apparent skewing of gender ratios in this group, suggesting that both genders were equally affected. The surviving double-KO mice demonstrated a retarded growth rate; first showing a significantly lower body weight compared to their mGPD-KO littermates by 36 days of age in males and 29 days in females (Fig. 1). The mGPD-KO littermates that were heterozygous for the Ctrn-KO mutation (Ctrn+/+, mGPD+/-) showed a similar growth rate to that of the mGPD-KO mice (Fig. 1). Comparisons between wild-type and Ctrn-KO mice also showed similar growth rates (42), and mGPD-KO mice showed only a small difference in growth rate compared to non-littermate wild-type mice (data not shown).

Hypoglycemia in Ctrn/mGPD double-KO mice - Figure 2 shows that both the Ctrn-KO and Ctrn/mGPD double-KO mice are hypoglycemic under fasted conditions when compared to wild-type mice, with the double-KO demonstrating a more severe form. The double-KO mice were also hypoglycemic under fed conditions (148 ± 33b mg/dl; n = 8) when compared with wild-type (222 ± 31a mg/dl; n = 11) and mGPD-KO (219 ± 35a mg/dl; n = 8) mice, with the Ctrn-KO mice again showing an intermediate phenotype (194 ± 49ab mg/dl; n = 7). Plasma insulin concentrations (ng/ml; n = 4) under fed conditions were significantly lower in Ctrn-KO (0.80 ± 0.68b) and double-KO (0.87 ± 0.40b) mice compared to wild-type (2.69 ± 0.82a) mice, with the mGPD-KO showing an intermediate (1.57 ± 1.03ab) level.

Hyperammonemia in Ctrn/mGPD double-KO mice and effect of oral sucrose administration - Under fasted conditions, Fig. 3A shows no difference in blood ammonia levels among mice of the four genotypes examined. Under fed conditions however, the double-KO mice
showed an elevated blood ammonia level (Fig. 3A). Since patients with citrin deficiency have been reported to dislike foods high in carbohydrates (2), with several patients experiencing bouts of hyperammonemia following carbohydrate administration (3), our unpublished data), we examined the effect of oral sucrose administration (10 g/kg) on blood ammonia levels. As shown in Fig. 3B, the double-KO mice showed hyperammonemia not only under fed conditions, but demonstrated a further two to three-fold increase in blood ammonia levels (~500 μg/dl) 60 min after the oral sucrose administration. No increase in blood ammonia levels was observed in the mGPD-KO and Ctrn-KO mice following the oral sucrose administration. Even under fasted conditions, oral sucrose and glucose (5 g/kg) administration resulted in an increased blood ammonia level in the double-KO mice (the 60-min values after administration: 419 ± 99 and 372 ± 122 μg/dl, respectively; n = 3).

Hepatic lactate/pyruvate ratio and effect of sucrose administration – Under the same conditions as those shown in Fig. 3B, we examined the hepatic L/P ratio as a measure of the cytosolic NADH/NAD⁺ ratio (54). As shown in Table 2, the hepatic L/P ratio was significantly higher in the double-KO mice compared to the mGPD-KO mice at zero time and 60 min following oral sucrose administration. The changes in the L/P ratio observed in the double-KO mice were mainly brought about by a decrease in the Pyr concentration. There was no significant change in the Lac and Pyr concentrations or the L/P ratio in mGPD-KO mice following oral sucrose administration.

Urea-cycle enzyme activities and hepatic Asp content - As an initial attempt to clarify the pathophysiology of the hyperammonemia observed in the double-KO mice, we measured the urea-cycle enzyme activities in the liver of the mice. As shown in Fig. 4, the ASS activity was not different in Ctrn-KO, mGPD-KO or double-KO mice compared to wild-type controls, although it was significantly lower in mGPD-KO mice compared with the double-KO mice. The double-KO mice showed only a slight increase in arginase activity and a slight decrease in CPS and OCT activities compared to wild-type controls however, the mGPD-KO mice also showed similar trends in activities for these enzymes that likely explain these differences. Finally, the ASL level was only marginally decreased in the mGPD-KO mice compared to wild-type controls.

We also assayed the hepatic Asp content (nmol/g liver) of wild-type, Ctrn-KO, mGPD-KO and Ctrn/mGPD double-KO mice and found that the double-KO mice showed a significantly (p < 0.05) lower level (373 ± 115b; n = 9) compared to wild-type (756 ± 52b; n = 6) and mGPD-KO (676 ± 136b; n = 8) mice, with the Ctrn-KO (539 ± 177ab; n = 6) mice showing an intermediate phenotype.

Plasma amino acid concentrations - Table 3 shows plasma amino acid and urea concentrations from fasted wild-type, Ctrn-KO, mGPD-KO and Ctrn/mGPD double-KO mice. Only the levels of Cit and the Thr/Ser ratio were significantly higher in the double-KO mice compared to the other genotypes, with other markers characteristic of human citrin deficiency such as an increase in Arg or a decrease in BCAA/AAA ratio, remaining unchanged. The Cit level and Thr/Ser ratio were also elevated under fed conditions in the
double-KO mice compared to the other genotypes, although the Thr/Ser ratio in the double-KO mice compared to the Ctrn-KO mice failed to reach statistical significance (data not shown). The plasma urea concentration of the double-KO mice was significantly higher than wild-type and Ctrn-KO mice and may be the result of increased protein degradation in the double-KO mice.

The oral sucrose administration did not cause any significant changes in plasma Cit levels or alterations in the Thr/Ser ratio (data not shown). The plasma Ala concentration and BCAA/AAA ratio of the double-KO mice were significantly decreased, however, following oral sucrose administration (Fig. 5); the former change being consistent with a decreased hepatic Pyr concentration (Table 2).

Fatty liver and related plasma parameters - Both Ctrn-KO and double-KO mice were found to have a fatty liver following a 16 h fast (Fig. 6). The liver TG content was found to be significantly higher in the Ctrn-KO and double-KO mice compared to that of wild-type and mGPD-KO mice. Table 4 shows plasma parameters related to lipid and fatty acid metabolism measured in fasted wild-type, Ctrn-KO, mGPD-KO and double-KO mice. The plasma TG concentrations were similar among the four genotypes, with the Ctrn-KO mice showing the highest while the mGPD-KO mice showing the lowest levels. The mGPD-KO mice showed a significantly higher cholesterol level compared to wild-type mice. The double-KO mice however, revealed significantly elevated plasma FFA and glycerol levels compared to the other genotypes, although the difference compared to the Ctrn-KO mice failed to reach statistical significance. Lastly there was no difference in ketone bodies or bile acid levels among the four genotypes.

Table 4 also shows plasma parameters related to liver damage in wild-type, Ctrn-KO, mGPD-KO and double-KO mice. The double-KO mice revealed a significantly elevated plasma ALP activity compared to mice of the other genotypes. The plasma ALT and AST activities of the double-KO mice failed to show any consistent increase above the levels of the other genotypes.

Microarray analysis of genes differentially expressed in Ctrn-KO, mGPD-KO and the double-KO mice - We assessed the differential expression levels of over 40,000 transcripts in livers from Ctrn-KO, mGPD-KO and double-KO mice compared to wild-type controls. There were 123, 29 and 102 genes that consistently demonstrated at least a 2-fold differential expression level in the liver of Ctrn-KO, mGPD-KO and double-KO mice, respectively (Fig. 7A; also shown in Supplemental Table). Of the 102 genes that were differentially expressed in the double-KO mice, 62 genes were unique to the double-KO mice compared to either the Ctrn-KO or mGPD-KO mice, 19 genes were in common with the Ctrn-KO mice, 17 were in common with the mGPD-KO mice, and 4 were shared among all three genotypes (Fig. 7A). All 102 genes differentially expressed in the livers of the double-KO mice are listed in Fig. 7B. Of special note was the relative expression level of aralar, the brain-/muscle-type AGC, that can functionally compensate for citrin (5). Aralar expression has previously not been detected in human liver by northern or western blot (3,4,6), and is most minimally detectable in mouse liver
based similar methods (7,42). The relative expression level of aralar in the liver of the double-KO mice, detected by microarray analysis, was only slightly higher (1.40 ± 0.19 folds) than that of wild-type mice.

Based on the microarray results, the mRNA levels of two highly expressed genes, flavin-containing monooxygenase 3 (Fmo3) and asparagine synthetase (Asns), two more moderately expressed genes related to fatty acid synthesis, ATP citrate lyase (Acly) and malic enzyme (Mod1), and glutamine synthetase (GluL) as a control were assayed relative to that of Gapdh using real-time RT-PCR analysis. Our results confirmed those found by the microarray analyses, with Fmo3 and Asns demonstrating more pronounced differential expression levels (Fig. 8).

DISCUSSION

As reported previously, Ctrn-KO mice analyzed on a mixed genetic background showed no apparent phenotype related to human citrin deficiency (either CTLN2 or NICCD) despite liver perfusion experiments revealing measurable deficits in ureogenesis from ammonia and gluconeogenesis from Lac, and an elevated L/P ratio (42,55) indicative of an altered cytosolic NADH/NAD⁺ ratio (54). In the present study, although we found that the Ctrn-KO mice do demonstrate mild hypoglycemia and a fatty liver when congenic on the C57BL/6J background, we hypothesized that the absence of a more severe phenotype in these mice was most likely due to a higher GP shuttle activity in mouse liver, which can compensate for the decreased MA shuttle activity resulting from the loss of the liver-type AGC, citrin. The mGPD-KO mice on a C57BL/6J background in the present study showed only mild effects on growth rate and no effect on blood glucose levels compared to wild-type controls in contrast to a previous report (56).

Our present study of the double-KO mice showed that, unlike the Ctrn-KO mice, they displayed numerous characteristics of human citrin deficiency. It is also important to point out however, that we observed a 30% decreased survival of Ctrn/mGPD double-KO mice by 20 to 40 days of age, and that all experiments performed made use of mice that survived to maturity. Although further investigations are needed to define the time-point and reason for this suspected lethality, it is conceivable that the mice may have succumbed to severe metabolic disturbances prior to, or just after, birth. This would be consistent with several NICCD patients who have displayed such severe symptoms that they required liver transplantation (40,41).

The most striking phenotype of the surviving Ctrn/mGPD double-KO mice related to human citrin deficiency was that of hyperammonemia under fed, but not fasted, conditions. This finding is consistent with diurnal fluctuations in the blood ammonia levels observed in some CTLN2 patients (34). Unlike most CTLN2 patients however, we did not detect any loss of ASS activity in the livers of the double-KO mice compared to wild-type, Ctrn-KO or mGPD-KO mice (nor any substantial changes in the activity of the other urea-cycle enzymes). We have shown previously that the decrease in hepatic ASS levels in CTLN2 patients can be variable, indicating that its loss is secondary to citrin
deficiency (16), which is further supported by the fact that no decrease in hepatic ASS activity has been observed in NICCD patients examined so far (35). Together with our present findings on the double-KO mice, the decrease of hepatic ASS seen in CTLN2 may not be the only mechanism leading to the development of hyperammonemia.

As a result of citrin deficiency, Asp cannot be supplied from mitochondria, and we have shown previously by liver perfusion that the cytosolic Asp concentration is rate-limiting in the Ctn-KO liver (42). Cytosolic Asp must therefore come from either the transamination of Glu to oxaloacetate via cytosolic AST that would be inhibited by the high cytosolic NADH/NAD\(^+\) ratio, or from the deamination of asparagine (Asn) via asparaginase reaction. This latter route is supported by our discovery of an increased expression of the Asns gene in the livers of Ctn-KO and double-KO mice by microarray and confirmation using real-time RT-PCR. Previous liver perfusion experiments revealed that when Asn is added to the perfusate, there is an increased rate of ureogenesis in the Ctn-KO mice (42) accompanied by an increased hepatic Asp and ammonia concentrations (55), suggesting that Asn is stimulating ureogenesis through its conversion to Asp and ammonia. This was not true of added Asp under similar conditions (55) owing to its impermeability into periportal hepatocytes (57). A survey of various mammalian and avian species has shown that human and mouse liver have comparable asparagine synthetase activities, but that mouse liver has a thousand-fold increased activity of asparaginase compared to that of human (58). An increased Asn synthesis through the increased expression of the Asns gene in the Ctn-KO and the double-KO mice therefore could supply Asn as a diffusible source of cytosolic Asp within the mouse liver. Our present results revealed a 30-50% decrease in the level of hepatic Asp in the double-KO mice compared to the other genotypes, although it is possible that this value is artifically high owing to possible contamination from mitochondrial Asp stores as we did not separate hepatic amino acids into cytosolic and mitochondrial fractions. A more detailed examination is needed to clarify the extent to which the limited availability of Asp leads to the hyperammonemia observed in the Ctn/mGPD double-KO mice and whether intra- or inter-cellular Asn cycling within the mouse liver is a compensatory source of cytosolic Asp for ureogenesis.

Many patients with human citrin deficiency have been reported to have a peculiar fondness for foods high in protein and fat, and a dislike of foods high in carbohydrates (1,2,13,27). This is in contrast to CTLN1 patients or those with other urea-cycle disorders, where dietary intake of protein is typically restricted to prevent the build-up of ammonia from protein degradation (59). Furthermore, there have been several clinical reports of glucose, glycerol or fructose infusion as a therapy for hyperammonemia and brain edema in CTLN2 patients that resulted in an enhanced hyperammonemia and a rapid deterioration (31-33). Based on these clinical observations, we challenged the Ctn/mGPD double-KO mice with an oral sucrose solution and found a further increase in blood ammonia levels, confirming the observations made in the CTLN2 patients. The effect of the oral sucrose administration on blood ammonia levels in the double-KO mice
provides a likely explanation for why CTLN2 patients avoid foods high in carbohydrates. The increased L/P ratio also observed in the double-KO mice following oral sucrose administration (Table 2) suggests that the increased ammonia levels are closely associated with a further perturbation of the NADH/NAD$^+$ ratio from the generation of cytosolic NADH during glycolysis, and this altered redox state may depress urea-cycle enzyme activities by further limiting cytosolic Asp. A further altered redox state may also effect ATP production through limited availability of Pyr for oxidation within the mitochondria. Previous liver perfusion experiments have shown that the addition of either Pyr or phenazine methosulfate to the perfusate, substances that both act to reduced the NADH/NAD$^+$ ratio in the liver, effectively ameliorated the deficit in ureogenesis from ammonia in the Ctrn-KO mice (55). All of our findings to date support the notion that carbohydrates or other substances that may act to increase the cytosolic NADH/NAD$^+$ ratio in patients with human citrin deficiency should be restricted to minimize the risk of hyperammonemia.

Our present studies of the Ctrn/mGPD double-KO mice also revealed a number of other important characteristics of human citrin deficiency. The decreased growth rate observed in the Ctrn/mGPD double-KO mice is consistent with reports that many CTLN2 patients are thin, and most NICCD patients have shown low birth weight (60) and growth retardation (2,27,28,61). Elevations in plasma Cit and an altered Thr/Ser ratio, both consistent with CTLN2 and NICCD (2), were observed in the double-KO mice under fed and fasted conditions. Although the plasma BCAA/AAA ratio, a marker of CTLN2 (17), was not different among any of the groups of mice under either fed or fasted conditions, the administration of the oral sucrose solution decreased the ratio in the double-KO mice, along with a decrease in the Ala concentration. Fasting also led to an accumulation of TG in the liver of Ctrn-KO and Ctrn/mGPD double-KO mice which has been observed in both NICCD and CTLN2 patients. Finally, the double-KO mice also demonstrated hypoglycemia under fasted conditions when compared with the other genotypes (with the Ctrn-KO mice showing an intermediate phenotype). Although hypoglycemia is not one of the main symptoms of CTLN2, it has been observed in NICCD patients (2,27,62) and is also likely related to an elevated cytosolic NADH/NAD$^+$ ratio leading to decreased gluconeogenesis from reduced substrates and lower oxaloacetate levels required by phosphoenolpyruvate carboxykinase. Overall, it is quite intriguing that the double-KO mice appear to exhibit phenotypes consistent with both NICCD and CTLN2 at this time-point. A more detailed analysis of these mice at various ages, including the neonatal period, is needed to clarify the extent to which these mice are a model of both NICCD and CTLN2.

The fatty liver observed in both the Ctrn-KO and Ctrn/mGPD double-KO mice was also accompanied by an elevated level of plasma FFA and glycerol in the double-KO mice. The elevation of plasma glycerol in the double-KO mice could result from a decreased ability to metabolize glycerol due to the combination of a generalized loss of mGPD activity with the hepatic loss of the MA shuttle in the double-KO mice. Alternatively, these phenotypes may
point to an underlying impairment in hepatic fatty acid oxidation as high plasma FFA in conjunction with hepatic TG accumulation, hypoglycemia and enhanced protein degradation as suggested by the elevated plasma urea levels are all consistent with this interpretation. A similar state of impaired hepatic fatty acid oxidation has been hypothesized in CTLN2 patients based on lower plasma ketone bodies with high FFA levels (25,63). The elevated expression of fat metabolism-related genes such as the fatty acid translocase (Cd36) and the very low density lipoprotein receptor (Vldlr) in both the Ctrn-KO and the double-KO mice, as revealed by our microarray analyses, may be more related to the hepatic TG accumulation observed. Further investigations are needed to establish whether these phenomena are indeed integral components of the phenotype of the double-KO mice as a model of human citrin deficiency.

Although our microarray analyses were performed primarily to uncover genes that might be involved in the major symptoms of citrin deficiency, they also have the potential to identify pathways involved in additional conditions associated with human citrin deficiency, such as an increased incidence of hepatocellular carcinoma, pancreatitis, steatohepatitis and hyperlipidemia in CTLN2 patients (1,2,8,21-30). From our present results, we found profound changes in gene expression related to pathways involved in drug metabolism, fat metabolism, oxidative stress and cell growth in the liver of double-KO mice. Changes in such pathways may be closely related to the complications of CTLN2 through cell proliferation, oxidative stress and enhanced fat metabolism. Enhanced expression of genes related to the suppression of oxidative stress, such as glutathione S-transferase (Gsta1), quinine NAD(P) dehydrogenase (Nqo1) and Gadd45α, and depressed expression of hydroxyacid oxidase 1 (Hao 1) and aldehyde oxidase 1 homologue (AOH 1 homologue) may be compensatory adaptations of the double-KO mice against such possible oxidative stress. Furthermore, a number of genes related to cell proliferation, such as cyclin D1 (Cnd1) and annexin A2 (Anxa2), together with cancer-associated genes such as Niban and Cd63, were highly expressed. Finally, genes for drug-metabolizing enzymes such as Fmo3 and several cytochrome P-450 genes which also may be connected to carcinogenesis, were the most differentially expressed genes compared to wild-type. Further analyses are needed to determine whether specific genes and pathways differentially expressed in the Ctrn/mGPD double-KO mice play an important role in their phenotypic spectrum as a model of human citrin deficiency.

Through our present studies of the Ctrn/mGPD double-KO mice, we have been able to demonstrate that the mice are indeed a more accurate model of human citrin deficiency compared to the Ctrn-KO mice alone. Although we have focused on the role of liver in the double-KO mice in regards to human citrin deficiency, it is conceivable that extrahepatic tissues may also be contributing to the overall phenotype observed in the double-KO mice as both citrin and mGPD are constitutively knocked out in all tissues that express them. Our justification for focusing on the liver however, is based on the simple observation that liver transplantation appears to completely correct all defects present in CTLN2 patients.
(1,8,23,26,39), indicating that the liver is the most important for human citrin deficiency. Furthermore, previous studies of Ctrn-KO and mGPD-KO mice alone have both shown that they are grossly phenotypically normal under standard conditions (42,45), therefore only metabolically active tissues requiring high NADH shuttle activities would likely present with a defect. Aralar is known to be expressed in a wider range of mouse tissues than citrin (7,42) including the brain, skeletal muscle and heart, likely compensating for the loss of citrin function in tissues that express both citrin and aralar. Finally, no changes in aralar expression have been detected in the Ctrn-KO mice in tissues found to have the highest citrin expression by either northern or western blot (42), and no significant change in aralar expression was detected in the liver of the double-KO mice, which has been shown previously to exhibit minimal aralar expression. Based on all of our findings to date, tissues other than the liver are likely to play only a small role in the observed phenotype of the double-KO mouse, and given that there is no evidence for changes in aralar expression to compensate for the loss of citrin function, and based on the overlapping expression patterns of aralar and citrin, the liver is the tissue most likely affected in the double-KO mice and the most relevant tissue for a model of human citrin deficiency.

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**FOOTNOTES**

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The abbreviations used are: AAA, aromatic amino acid; Acly, ATP citrate lyase; AGC, aspartate-glutamate carrier; ALP, alkaline phosphatase; ALT, alanine aminotransferase; Anxa2, annexin A2; AOH, aldehyde oxidase; ASL, argininosuccinate lyase; Asns, asparagine synthetase; ASS, argininosuccinate synthetase; AST, aspartate aminotransferase; BCAA, branched-chain amino acid; Ccnd1, cyclin D1; Cd36, fatty acid translocase; Cit, citrulline; CPS, carbamoylphosphate synthetase; CT, cycle threshold number; CTLN2, adult-onset type II citrullinemia; Ctrn, citrin; Cy3, cyanine 3-dCTP; Cy5, Cyanine 5-dCTP; FFA, free fatty acid; Fmo3, flavin-containing monooxygenase 3; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Glul, glutamine synthetase; GP, glycerol phosphate; Gsta1, glutathione S-transferase; Hao 1, hydroxyacid oxidase 1; Lac, lactate; L/P, lactate/pyruvate; KO, knockout; MA, malate-aspartate; mGPD, mitochondrial glycerol 3-phosphate dehydrogenase; Mod1, malic enzyme; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Nqo1, quinine NAD(P) dehydrogenase; OCT, ornithine carbamoyltransferase; PSTI, pancreatic secretory trypsin inhibitor; Pyr, pyruvate; TG, triglyceride; Vldlr, very low density lipoprotein receptor.
Figure legends

Fig. 1. Growth rates for male (left panel) and female (right panel) mGPD-KO (open circle), mGPD-KO mice heterozygous for Ctn (grey) and Ctn/mGPD double-KO (black) mice. The number of mice used was between 5 and 15 for each time-point. The values are expressed as means ± SD. An asterisk (*) and double asterisk (**) indicate a significant difference of Ctn/mGPD double-KO mice compared to mGPD-KO mice and mGPD-KO mice heterozygous for Ctn (Ctn⁻/⁺, mGPD⁻/⁻) at a significance level of *p < 0.05 and **p < 0.01 level, respectively.

Fig. 2. Plasma glucose concentrations of fasted wild-type, Ctn-KO, mGPD-KO and double-KO mice. The number of mice used was 14, 12, 9 and 9 for wild-type (W), Ctn-KO (C), mGPD-KO (G) and Ctn/mGPD double-KO (D) mice, respectively. The group means (± SD) were compared using a one-way ANOVA followed by the Tukey-Kramer test (alpha level = 0.05). Mean values sharing identical superscripts are not significantly different.

Fig. 3. Blood ammonia concentrations of fasted (dark column) and fed (grey column) wild-type (W), Ctn-KO (C), mGPD-KO (G) and Ctn/mGPD double-KO (D) mice in [A], and effect of sucrose administration on blood ammonia concentrations of Ctn-KO (square), mGPD-KO (triangle) and double-KO (circle) mice in [B]. Cardiac blood was taken under anesthesia between 10-11 a.m. from fed or fasted (for 16-17 h) mice in [A]. In [B], sucrose (10 g/kg; black) was administered per os to mice with a gastric tube at zero time-point (9-10 a.m.). Blood was taken from separate sets of mice for the initial time-point (white) and each subsequent time-point (black) indicated. The number of animals used is shown in parentheses in [A] while 5 to 8 mice were used for each time point in [B]. The means (± SD) were compared with a one-way ANOVA followed by the Tukey-Kramer test between four genotypes in [A] and between time points in [B]. Values sharing identical alphabetical superscripts are not significantly different. The pound sign (#) indicates a significant level of p < 0.01 (by Student’s t test).

Fig. 4. Urea-cycle enzyme activities in the liver of fed wild-type, Ctn-KO, mGPD-KO and Ctn/mGPD double-KO mice. Livers were taken from fed mice sacrificed by cervical dislocation between 9-11 a.m. Preparation of liver extracts and assay methods are described in the Experimental Procedures. The number of mice used was between 8 and 10 for each genotype. The means (± SD) were compared with a one-way ANOVA followed by the Tukey-Kramer test between each pairwise genotype group. Values sharing identical alphabetical superscripts are not significantly different. CPS, carbamoylphosphate synthetase; OCT, ornithine carbamoyltransferase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase.
Fig. 5. Changes in plasma alanine (Ala) concentration and BCAA/AAA ratio of fed mGPD-KO (triangle) and Ctrn/mGPD double-KO mice (circle) following sucrose administration. Experimental conditions were the same as in the legend of Fig. 3B. Amino acid levels were measured at the zero time-point (white) and 60 min following either an oral sucrose (dark) or sodium chloride (grey) administration. The number of mice used for each group was 8, with the exception of the 60 min time-point for the double-KO mice administered with sodium chloride and the mGPD-KO mice administered sucrose (n = 5). The pound sign (#) indicates a significant level of $p < 0.05$ (by Student’s $t$-test).

Fig. 6. Hepatic triglyceride concentration of fasted wild-type (W), Ctrn-KO (C), mGPD-KO (G) and double-KO (D) mice. Livers were taken from the mice fasted from 5 p.m. on the day prior to performed experiments for 16-17 h. Hepatic lipid was extracted as described in the Experimental Procedures. The number of mice used was 6 to 8 for each genotype. The means ($\pm$ SD) were compared with a one-way ANOVA followed by Tukey-Kramer test between all four genotypes. Values sharing identical alphabetical superscripts are not significantly different.

Fig. 7. Microarray analyses of liver gene expression levels from mGPD-KO, Ctrn-KO and double-KO mice compared to wild-type mice. [A] shows a Venn diagram of the number of genes that consistently demonstrated more than a 2-fold differential expression level in the liver of mGPD-KO, Ctrn-KO and double-KO mice compared to the wild-type reference, and the number of genes that overlap among the three groups. [B] shows a comparison of the fold-changes in gene expression among mGPD-KO, Ctrn-KO and Ctrn/mGPD double-KO mice for those genes found to significantly different between the double-KO and the wild-type reference. Gene expression levels are shown on a log2 scale compared to wild-type. The number of mice tested was 4 for each genotype.

Fig. 8. Quantitative real-time RT-PCR analysis of flavin-containing monooxygenase 3 (Fmo3), asparagine synthetase (Asns), glutamine synthetase (Glul), ATP citrate lyase (Acly), and malic enzyme (Mod1) mRNA from the livers of wild-type, Ctrn-KO (white), mGPD-KO (grey) and Ctrn/mGPD double-KO (dark) mice. Gene expression ratios were determined by real-time PCR. The wild-type values are set at 1 and the values are expressed on a log2 ratios. The number of mice used was 3 for each genotype. Values expressed are means $\pm$ SD.
Table 1. Primer pairs used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmo3</td>
<td>F: 5’-GGGCCTGTGGAAATTCTCA-3’&lt;br&gt;R: 5’-TTGGTGAAGACCGATTGGTAAA-3’</td>
<td>69</td>
</tr>
<tr>
<td>Asns</td>
<td>F: 5’-GGAGGACAGCCCCGATCT-3’&lt;br&gt;R: 5’-TTCAATGATGCTCCTCCCAA-3’</td>
<td>67</td>
</tr>
<tr>
<td>Glul</td>
<td>F: 5’-GAGAAAGGACTGCGCTGCAA-3’&lt;br&gt;R: 5’-CCACTCAGGTAACTCTCCACACA-3’</td>
<td>71</td>
</tr>
<tr>
<td>Acly</td>
<td>F: 5’-CATCGGCCATCGAGTAAAATC-3’&lt;br&gt;R: 5’-TGCTGTTTGACGAAGTCCTGA-3’</td>
<td>75</td>
</tr>
<tr>
<td>Mod1</td>
<td>F: 5’-CTGCAGACGAGTGCTACAAGGT-3’&lt;br&gt;R: 5’-TGGAAGAGTGACTGGATCAAAAG-3’</td>
<td>80</td>
</tr>
<tr>
<td>Gapdh</td>
<td>F: 5’-ATGGTGAAAGGTCGGTGTA-3’&lt;br&gt;R: 5’-GAGTGAGCTCATACTGGAAC-3’</td>
<td>151</td>
</tr>
</tbody>
</table>

Fmo3, flavin-containing monooxygenase 3; Asns, asparagine synthetase; Glul, glutamine synthetase; Acly, ATP citrate lyase; Mod1, malic enzyme; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.
Table 2. Effect of sucrose administration on lactate and pyruvate concentration and lactate/pyruvate (L/P) ratio in the liver of mGPD-KO and Ctn/mGPD double-KO mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>n</th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>L/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-time</td>
<td>mGPD-KO</td>
<td>8</td>
<td>800 ± 450</td>
<td>66 ± 16</td>
<td>13 ± 8</td>
</tr>
<tr>
<td></td>
<td>Double-KO</td>
<td>5</td>
<td>1300 ± 200*</td>
<td>42 ± 21*</td>
<td>36 ± 14**</td>
</tr>
<tr>
<td>Sucrose</td>
<td>mGPD-KO</td>
<td>6</td>
<td>1150 ± 360</td>
<td>66 ± 20</td>
<td>20 ± 9</td>
</tr>
<tr>
<td></td>
<td>Double-KO</td>
<td>5</td>
<td>1720 ± 550</td>
<td>21 ± 9** #</td>
<td>75 ± 37#</td>
</tr>
</tbody>
</table>

Experimental procedures are as described in the legend of Fig. 3B.

*p<0.05; **p<0.01 vs mGPD-KO mice. #p<0.01 vs zero-time mice.
Table 3. Comparison of plasma amino acids among fasted wild-type, Ctn-KO, mGPD-KO and double-KO mice (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>Ctn-KO</th>
<th>mGPD-KO</th>
<th>Double-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>9450 ± 2180b</td>
<td>8190 ± 720b</td>
<td>11100 ± 1690ab</td>
<td>14900 ± 4220a</td>
</tr>
<tr>
<td>Asp</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Thr</td>
<td>133 ± 17ab</td>
<td>111 ± 17b</td>
<td>109 ± 17b</td>
<td>146 ± 25a</td>
</tr>
<tr>
<td>Ser</td>
<td>75 ± 8</td>
<td>66 ± 4</td>
<td>61 ± 9</td>
<td>69 ± 13</td>
</tr>
<tr>
<td>Asn</td>
<td>30 ± 3ab</td>
<td>35 ± 6ab</td>
<td>28 ± 7b</td>
<td>38 ± 5a</td>
</tr>
<tr>
<td>Glu</td>
<td>12 ± 4</td>
<td>20 ± 7</td>
<td>15 ± 5</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>Gln</td>
<td>710 ± 42a</td>
<td>649 ± 85ab</td>
<td>587 ± 55b</td>
<td>681 ± 43ab</td>
</tr>
<tr>
<td>Gly</td>
<td>147 ± 17</td>
<td>144 ± 21</td>
<td>135 ± 16</td>
<td>130 ± 32</td>
</tr>
<tr>
<td>Ala</td>
<td>221 ± 24</td>
<td>223 ± 38</td>
<td>203 ± 42</td>
<td>236 ± 29</td>
</tr>
<tr>
<td>Cit</td>
<td>51 ± 6b</td>
<td>52 ± 9b</td>
<td>64 ± 13b</td>
<td>88 ± 20a</td>
</tr>
<tr>
<td>Val</td>
<td>191 ± 48ab</td>
<td>139 ± 14b</td>
<td>203 ± 41a</td>
<td>183 ± 24ab</td>
</tr>
<tr>
<td>Cys</td>
<td>23 ± 3</td>
<td>21 ± 9</td>
<td>20 ± 8</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>Met</td>
<td>51 ± 8</td>
<td>42 ± 6</td>
<td>42 ± 6</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>Ile</td>
<td>80 ± 18ab</td>
<td>64 ± 13b</td>
<td>95 ± 14a</td>
<td>85 ± 23ab</td>
</tr>
<tr>
<td>Leu</td>
<td>147 ± 36ab</td>
<td>110 ± 17b</td>
<td>158 ± 23a</td>
<td>149 ± 28ab</td>
</tr>
<tr>
<td>Tyr</td>
<td>44 ± 14</td>
<td>42 ± 14</td>
<td>61 ± 14</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>Phe</td>
<td>73 ± 8</td>
<td>59 ± 4</td>
<td>69 ± 15</td>
<td>69 ± 15</td>
</tr>
<tr>
<td>Orn</td>
<td>34 ± 8ab</td>
<td>31 ± 5b</td>
<td>37 ± 6ab</td>
<td>42 ± 4a</td>
</tr>
<tr>
<td>His</td>
<td>47 ± 5ab</td>
<td>44 ± 3b</td>
<td>46 ± 5ab</td>
<td>54 ± 8a</td>
</tr>
<tr>
<td>Lys</td>
<td>215 ± 29a</td>
<td>150 ± 16b</td>
<td>163 ± 18ab</td>
<td>178 ± 43ab</td>
</tr>
<tr>
<td>Trp</td>
<td>80 ± 24</td>
<td>72 ± 27</td>
<td>77 ± 23</td>
<td>67 ± 22</td>
</tr>
<tr>
<td>Arg</td>
<td>61 ± 10</td>
<td>52 ± 5</td>
<td>53 ± 11</td>
<td>55 ± 13</td>
</tr>
<tr>
<td>Pro</td>
<td>47 ± 14</td>
<td>63 ± 21</td>
<td>42 ± 12</td>
<td>47 ± 18</td>
</tr>
<tr>
<td>Thr/Ser</td>
<td>1.78 ± 0.11b</td>
<td>1.70 ± 0.27b</td>
<td>1.80 ± 0.16b</td>
<td>2.11 ± 0.12a</td>
</tr>
<tr>
<td>BCAA/AAA</td>
<td>3.53 ± 0.42</td>
<td>3.17 ± 0.67</td>
<td>3.59 ± 0.76</td>
<td>3.40 ± 0.77</td>
</tr>
</tbody>
</table>

Cit and Orn indicate citrulline and ornithine. BCAA and AAA represent the sum of Val, Ile and Leu, and the sum of Phe and Tyr, respectively. The values (nmol/ml), expressed as means ± SD, were compared with a one-way ANOVA followed by the Tukey-Kramer test (alpha level = 0.05). Values sharing identical superscripts are not significantly different.
Table 4. Comparison of plasma parameters among fasted wild-type, Ctrn-KO, mGPD-KO and double-KO mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type (n)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>38 ± 22 (8)(^{ab})</td>
</tr>
<tr>
<td>FFA (μEq/L)</td>
<td>464 ± 180 (8)(^{b})</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>64 ± 10 (14)(^{b})</td>
</tr>
<tr>
<td>Glycerol (nmol/ml)</td>
<td>133 ± 40 (8)(^{b})</td>
</tr>
<tr>
<td>Ketone bodies (μmol/L)</td>
<td>1260 ± 420 (8)</td>
</tr>
<tr>
<td>Bile acid (μmol/L)</td>
<td>8 ± 2 (6)</td>
</tr>
<tr>
<td>AST (KU)</td>
<td>75 ± 27 (14)</td>
</tr>
<tr>
<td>ALT (KU)</td>
<td>20 ± 7 (14)(^{ab})</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>70 ± 10 (6)(^{b})</td>
</tr>
</tbody>
</table>

The values, expressed as means ± SD, were compared with a one-way ANOVA followed by the Tukey-Kramer test (alpha level = 0.05). Values sharing identical superscripts are not significantly different.
Fig. 1
Fig. 2

Plasma Glucose (mg/dl)
Fig. 3
Fig. 4
Fig. 5
Fig. 6

Hepatic Triglyceride (mg/g liver)

W  C  G  D

0  20  40  60  80  100  120  140
Fig. 7

[A]

mGPD-KO mice  Ctrn-KO mice  Double-KO mice

7  1  99
4  17  19
62

Log2 ratio

mGPD-KO  Ctrn-KO  Double-KO

[A]

mGPD-KO mice  Ctrn-KO mice  Double-KO mice

7  1  99
4  17  19
62

Log2 ratio

mGPD-KO  Ctrn-KO  Double-KO

[A]

mGPD-KO mice  Ctrn-KO mice  Double-KO mice

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Log2 ratio

mGPD-KO  Ctrn-KO  Double-KO

[A]

mGPD-KO mice  Ctrn-KO mice  Double-KO mice

7  1  99
4  17  19
62

Log2 ratio
Fig. 8
Citrin/mitochondrial glycerol 3-phosphate dehydrogenase double-knockout mice recapitulate features of human citrin deficiency
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