TABLE 4

The fraction of pyruvate carboxylated is low in human islets as judged from the $^{14}CO_2$ ratios method

The [2-^{14}C]pyruvate/[3-^{14}C]pyruvate CO_2 ratio was calculated by dividing the $^{14}CO_2$ released from [2-^{14}C]glucose by that from [6-^{14}C]glucose, and the [1-^{14}C]acetate/[2-^{14}C]acetate ratio was calculated by dividing the $^{14}CO_2$ released from dimethyl [1,4-^{14}C]succinate by that from dimethyl [2,3-^{14}C]succinate (14–16). The fraction of pyruvate carboxylated expressed as a percent of pyruvate carboxylation plus pyruvate decarboxylation was calculated from the pyruvate and acetate ratios using the formula described under "Experimental Procedures." The ratios were calculated assuming 80% (the lower estimate) and 100% randomization (the higher estimate) of oxaloacetate carbon in the combined malate dehydrogenase and fumarase reactions prior to metabolism in the citric acid cycle. Results of CO_2 formation are shown as the mean ± S.E. (*N*) of data from experiments with *N* batches of rat islets or batches of human islets from *N* donors. In each experiment, there were 3 or 4 replicate test tubes of rat islets and 6 replicate test tubes of human islets (100 islets/test tube for each condition). The mean ± S.E. BMI and age of the human islet donors whose islets were used for these measurements were 27.7 ± 1.5 kg/m^2 and 46.9 ± 4.2 years.

Islets	Tracer	CO_2 formation	CO_2 ratios	Pyruvate carboxylation
		nmol $^{14}CO_2$/90 min		*%*
Rat	[2-^{14}C]Glucose	2.33 ± 0.33 (5)	Pyruvate $^{14}CO_2$ ratio	
Rat	[6-^{14}C]Glucose	1.29 ± 0.16 (5)	1.81	
Rat	Dimethyl [1,4-^{14}C]succinate	4.30 ± 0.57 (3)	Acetate $^{14}CO_2$ ratio	
Rat	Dimethyl [2,3-^{14}C]succinate	0.92 ± 0.45 (3)	4.67	46–55
Human	[2-^{14}C]Glucose	1.85 ± 0.19 (7)	Pyruvate $^{14}CO_2$ ratio	
Human	[6-^{14}C]Glucose	1.12 ± 0.11 (7)	1.65	
Human	Dimethyl [1,4-^{14}C]succinate	2.32 ± 0.41 (7)	Acetate $^{14}CO_2$ ratio	
Human	Dimethyl [2,3-^{14}C]succinate	1.26 ± 0.34 (7)	1.84	14–17

TABLE 5

The ratio of pancreatic islet PC mRNA to liver PC mRNA is much lower in the human than in the rat

mRNA levels were estimated by quantitative PCR. The level of PC mRNA was divided by the levels of various other mRNAs in the same tissue. The islet ratio was then divided by the same ratio in liver, and the islet value was expressed as a percentage of the value from liver of the same species with the average values from two human livers and four rat livers each set at 100%. Two primer sets were used for human liver. Results are the mean ± S.E. of islets from nine human donors and four batches of rat islets. The mean ± S.E. BMI and age of the human donors whose islets were used for these measurements were 30 ± 1.8 kg/m^2 and 46.3 ± 5.4 years. FAS is fatty-acid synthase.

PC mRNA/other mRNA	Ratio of human islet PC mRNA to liver PC mRNA		Ratio of rat islet PC mRNA to liver PC mRNA	Ratio of rat islet to human islet
	PC1 primers	PC2 primers		
	% of liver		*% of liver*	*fold*
PC/Glud1	8.9 ± 0.9	15.0 ± 3.2	73 ± 7	6.1
PC/ME1	1.6 ± 0.3	3.0 ± 0.4	9 ± 1.9	3.9
PC/mGPD	0.4 ± 0.1	0.7 ± 0.1	3.9 ± 0.3	7.1
PC/Idh1	14 ± 2.6	27 ± 6.0	216 ± 28	10.5
PC/Idh3a	1.6 ± 0.4	2.9 ± 0.6	8.6 ± 1.3	3.8
PC/FAS	5.2 ± 0.1	9.7 ± 1.5	41 ± 8	5.5

tive ratios for human islets, the average of these ratios in rat islets was 6.2 times their average in human islets (range 3.8–10.5). This suggests that the low level of PC in human islets is a result of a low level of transcription of the PC gene.

Low PC in Human Islets Is Intrinsic to the Islets—Our surprising observation of low PC in human islets demands that we eliminate all possible reasons to explain why the low PC could be an artifact of islet handling or donor conditions even though an artifact would seem unlikely because, as shown in Table 6, glucose-stimulated insulin release in these islets is robust.

Islet Donor Conditions—The low PC is not likely explained by the long term conditions or short term medical management of the islet donors because there were numerous different causes of death, and some died after an illness, and some died suddenly. Because obesity is diabetogenic by causing insulin resistance and can directly influence the condition of an individual's islet beta cells, the possible correlation of BMIs of the islet donors with PC activity was considered. Also Pietilainen *et al.* (49) recently reported down-regulation of PC mRNA transcripts in adipose tissue of the obese twin of twin pairs discordant for obesity. In our study, the body mass index of the human donors ranged from 20 to 51 kg/m^2, but islet PC activity showed no relationship with body mass index, as judged from linear regression analysis (correlation coefficient $r = 0.1$; see supplemental Fig. 1). (Indeed, the person with a BMI of 51 kg/m^2 had one of the highest islet PC activities (10.1 nmol of CO_2 fixed per min/mg of protein).) There was also no correlation of PC activity or PC protein with age or gender of the donors.

TABLE 6

The magnitude of glucose-stimulated insulin release in human islets is similar to rat islets and β-hydroxybutyrate potentiates glucose-stimulated insulin release

As described under "Experimental Procedures," islets were incubated with a submaximal stimulatory concentration of glucose (5.6 mM) with or without β-hydroxybutyrate (HB) for 1 h. Stimulation with 16.7 mM glucose, which provides a near maximal insulin release, is shown as a positive control. Results are from up to six experiments with 5–6 replicates for each condition in each experiment with islet preparations from six human donors and are the mean ± S.E. with the number of replicates in parentheses. The mean ± S.E. BMI and age of the human islet donors whose islets were used for these measurements were 28 ± 2.4 kg/m^2 and 49.8 ± 4.3 years. Insulin release from up to 6 batches of rat islets with 6–12 replicates for each condition are shown to demonstrate that the magnitude of glucose-induced insulin release from human islets is similar to that from rat islets. Insulin release is expressed in microunits of insulin/μg islet protein/1 h.

Secretagogue	Insulin release	
	Human islets	Rat islets
No addition	16 ± 1 (36)	12 ± 0.5 (36)
Glucose (5.6 mM)	60 ± 4 (36)[a]	57 ± 4 (24)[a]
Glucose (5.6 mM) + HB (5 mM)	128 ± 8 (36)[b]	102 ± 12 (33)[b]
Glucose (5.6 mM) + HB (1 mM)	91 ± 8 (18)[c]	
HB (5 mM)	21 ± 3 (12)	30 ± 2 (37)
Glucose (16.7 mM)	263 ± 23 (33)[a]	273 ± 25 (48)[a]

[a] $p < 0.001$ is *versus* no addition.
[b] $p < 0.001$ is *versus* glucose (5.6 mM).
[c] $p < 0.002$ is *versus* glucose (5.6 mM).

PC Protein Stability—The lower PC enzyme activity and PC protein in human pancreatic islets compared with rodent islets and clonal cell lines cannot be explained by rapid degradation of the human PC protein during the islet isolation procedure or more rapid loss of PC activity in human islets (compared with rodent islets or INS-1 832/13 cells) during frozen storage of tissue. We did notice that PC enzyme activity in human islets or

rat islets and other tissues decreased extensively with repeated freezing and thawing of frozen cell homogenates; therefore, we have reported measurements of PC enzyme activity on only freshly prepared homogenates of tissue that had either not been frozen or on the first thaw of a homogenate stored for about 1 day. However, repeated freezing and thawing of tissue homogenates and length of frozen storage of the islets and other tissues did not affect the level of PC protein.

The PC protein was not low in human liver after 7 months of frozen storage (Fig. 3). However, the PC level of protein in human islets analyzed by streptavidin blotting after intervals of a few hours to 30 days and even up to 8–10 years of frozen storage was extremely low, whereas the level of the PC protein in rat islets and mouse islets stored frozen for similar lengths of time and also for up to 10 years was not low (Fig. 2). In the rat, the level of PC in islets is similar to the PC levels in liver or kidney (Fig. 3) (4), whereas in the human, the level of the PC protein is lower in the islet than in liver (Fig. 3).

Islet Purity, Composition, and Viability—To explain the low PC in human islets on the basis of low islet purity or low viability, the purity and/or viability would need to equal 10–15%, an implausibly low number. In addition, as mentioned above, the activity of the mitochondrial enzyme mGPD, which is as highly expressed in human islets as in rat islets and mouse islets (31, 43–47) and is even more beta cell-specific than PC, was as high in the human islets as in the rat islets (Table 3). This indicates the purity and percentage of beta cells of the human islets were similar to those of the rat islets. In addition, the relative rate of pyruvate carboxylation was low in intact human islets compared with intact rat islets (Table 4). For these experiments, islets were selected manually under a dissecting microscope, and therefore, purity should have been excellent for both human and rat islets. Regarding the cell composition of human islet preparations, recent analyses by experts in this field indicate that the percentage of beta cells in human islets is similar to rodent islets (50, 51). Furthermore, the low human islet PC cannot be explained by poor islet quality. Some of the highest quality human islets as judged by high ATP/ADP ratios and high ratios of glucose-stimulated insulin release to unstimulated insulin release possessed some of the lowest PC activities (see supplemental material).

Is There Down-regulation of PC and ATP Citrate Islets during Preparation of Human Islets?—If low PC and ATP citrate lyase is not a characteristic of human islets *in vivo*, then down-regulation of these enzyme during the preparation and tissue culture of the islets would seem the most likely explanation for the low levels of these enzymes. However, if this is the case, then it appears that isolated human islets operate normally with low PC and low ATP citrate lyase because they secrete insulin as well as rat islets (Table 6) (13, 27) and metabolize glucose to similar extents (13–17, 52). PC activity did not change in human islets maintained in RPMI 1640 medium containing 5 mM glucose for 2 or 24 h, identical to the medium used for rat islets, which showed high PC levels instead of CMRL medium or PIM medium, which also contain 5 mM glucose.

Our quantitative PCR data (Table 5) and our mRNA microarray data (25), as well as microarray data found in databases (53, 54),[4] show that PC mRNA is low in comparison with other mRNAs in isolated human islets. It is likely the PC mRNA is low in human islets before the islet isolation procedure has begun. Weir[5] has noticed that the level of PC mRNA is very low compared with many other mRNAs in beta cells of islets of human pancreas analyzed *in situ* by laser capture microdissection (which, of course, does not involve islet isolation). Thus, it is likely that the cause of the low PC in the human beta cell is due to a low rate of transcription of the PC gene *in vivo*.

mGPD is regulated similarly to PC in rodent islets and clonal cell lines. Its transcription is influenced by some of the same transcription factors that influence the transcription of PC (8, 55–57), and both PC and mGPD are down-regulated in human islets isolated from subjects with type 2 diabetes (25), similarly to islets isolated from rodent models of type 2 diabetes (43, 58–60, 62). Because the level of mGPD is as high in normal human islets as in normal rat islets (Table 3), this is consistent with the idea that the low PC in human islets is specific for human islets.

SCOT Is Plentiful in Human Islets—The level of SCOT protein in human islets was much higher than in the INS-1 832/13 cell line and about twice as high as in rat islets and mouse islets as judged from immunoblot analysis (Fig. 6).

Acetoacetyl-CoA Synthetase Is Plentiful in Human Islets—The level of acetoacetyl-CoA synthetase protein in human islets and INS-1 832/13 cells was much higher than in rat islets and mouse islets as judged by immunoblot analysis (Fig. 7).

Islet Thiolases—Previous immunoblot analysis and estimates of specific mRNAs showed that pancreatic islets possess thiolases (26) that can reversibly convert acetyl-CoA to acetoacetyl-CoA. The levels of the proteins of the mitochondrial thiolase, acetyl-CoA acetyltransferase 1 (ACAT1), and the cytosolic thiolase, acetyl-CoA acetyltransferase 2 (ACAT2), were about the same in human islets as in rat pancreatic islets, liver, and kidney (Fig. 5).

Fatty-acid Synthase—The level of fatty-acid synthase protein in human islets was higher than in rat islets and equal to that in INS-1 832/13 cells also as judged by immunoblot analysis (Fig. 8).

Higher Acetoacetate and Lower Malate Levels in Glucose-stimulated Human Islets than in Rat Islets—Human islets and rat islets were incubated with a concentration of glucose that stimulates insulin secretion for 30 min, a time interval that includes the first phase of insulin secretion plus the initial part of the second phase of insulin secretion, which is the phase associated with metabolism of insulin secretagogues. Acetoacetate (Fig. 9) increased to a higher level in the human islets than in the rat islets, and malate (Fig. 10) increased to a lower level in the human islets than in the rat islets as judged by alkali-enhanced fluorescence assays.

In six samples of human islets maintained in CMRL or PIM medium prior to being washed and incubated in Krebs-Ringer bicarbonate buffer for 30 min, the fluorescence was extremely high. CMRL and PIM media contain NAD and NADP, which increased the fluorescence background and thus the measured

[4] GEO Database accession number GSE2060 is from Ref. 53.
[5] Gordon Weir, personal communication.

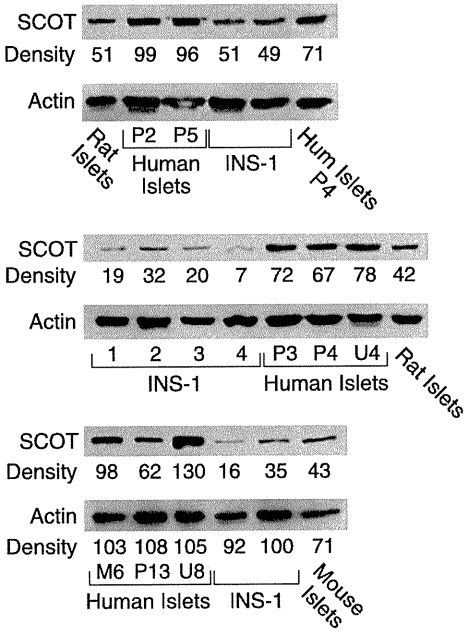

FIGURE 6. **Level of SCOT protein is higher in human islets than in rat islets and INS-1 832/13 cells.** Immunoblots were probed with anti-SCOT antibody. *Top panel,* 13 μg (*lane 6*) or 20 μg of whole-cell protein/lane (*other lanes*). (*Hum* is human islets.) *Middle panel,* immunoblot with 15 μg of whole-cell protein/lane. *Bottom panel,* 10 μg of whole-cell protein. The densities of the SCOT protein bands in each panel are expressed relative to the bands within the same panel. Membranes were stripped of antibody and reprobed with anti-β-actin antibody to discern relatively equal loading of protein across the lanes. In the *bottom panel,* due to the lower protein loaded in the lane of mouse islets, densities of the actin bands are also shown. The ratio of the relative density of the SCOT band to the actin band in the mouse lane shows the level of SCOT in mouse islets is about one-half the level in human islets, similar to in rat islets.

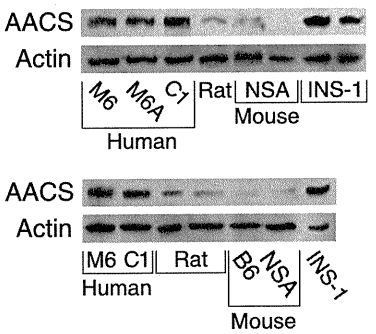

FIGURE 7. **Higher levels of acetoacetyl-CoA synthetase (*AACS*) protein in human pancreatic islets than in rat and mouse islets.** Two immunoblots with 15 μg of whole-cell protein/lane. The level of the enzyme (acetoacetyl-CoA synthetase) is much higher in human islets and INS-1 832/13 cells than in Sprague-Dawley rat islets and islets from the NSA (CF-1) mouse and the C57BL/6 (B6) mouse. Membranes were stripped of antibody and reprobed with anti-β-actin antibody to discern equal loading of protein across the lanes. A human islet lane that showed extremely dark staining was deleted from both panels.

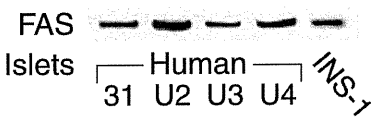

FIGURE 8. **High level of fatty-acid synthase protein in human islets.** *Immunoblot,* 15 μg of whole-cell protein/lane. The level of fatty-acid synthase (*FAS*) in human islets is as high as in INS-1 832/13 cells and much higher than in rat islets. The level of fatty-acid synthase in rat islets in this same blot was not visible, and the lane with rat islet protein is not shown.

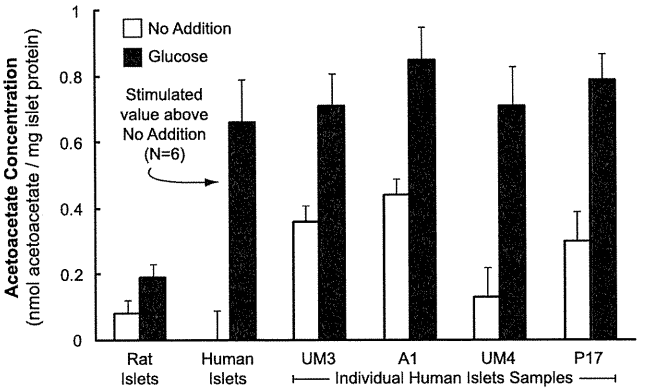

FIGURE 9. **Higher glucose-stimulated acetoacetate in glucose-stimulated human islets than in rat islets.** Islets were maintained in tissue culture medium containing 5 mM glucose for 4 h (some human) to 24 h (some human and all rat), washed, and then incubated in Krebs-Ringer bicarbonate buffer, pH 7.35, in the presence or absence of 16.7 mM glucose for 30 min. The figure shows the mean ± S.E. acetoacetate levels from eight separate experiments with 3–4 replicates of 100 rat islets for each condition (*left side* of figure) and 4–6 replicates of 100–200 islets for each condition from six separate experiments with islets from human donors (P11, C1, UM1, 258, P14, and P15) (*middle* of figure). Because of the high background fluorescence in the experiments with these six preparations of human islets that were shipped and maintained in media (CMRL or PIM) that contain NAD and NADP (see "Results"), which increased measured acetoacetate values to unrealistic high values, the no addition value for these experiments was assigned zero, and the glucose-stimulated increase in acetoacetate above the control is shown. Results of individual experiments with islets from four additional human donors are shown on the *right side* of the figure. For these experiments, to lower the background fluorescence, islets were maintained in RPMI 1640 tissue culture medium (modified to contain 5 mM glucose) for 24 h before incubation in the presence or absence of 16.7 mM glucose for 30 min as described above. [a], $p < 0.01$, or [b], $p < 0.001$ glucose-stimulated human islets *versus* glucose-stimulated rat islets. The mean ± S.E. BMI and age of the human islets donors whose islets were used for these measurements were 26.2 ± 1.6 kg/m^2 and 41.7 ± 5.3 years.

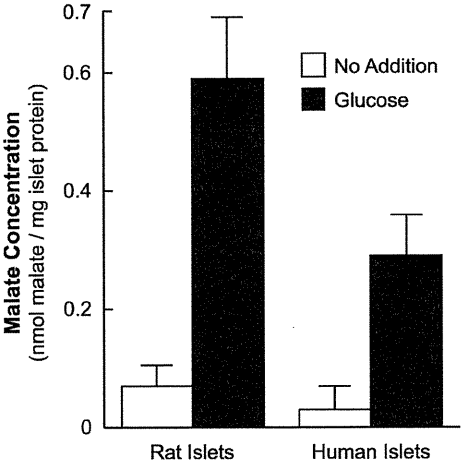

FIGURE 10. **Lower glucose-stimulated malate in human islets than in rat islets.** Malate was measured in the extracts from the experiments described in Fig. 9. Results are the mean ± S.E. of three experiments with rat islets and eight experiments with islets from human islets donors. [a], $p = 0.05$ glucose-stimulated human islets *versus* glucose-stimulated rat islets.

acetoacetate levels to unrealistically high values in the alkali-enhanced fluorescence assay for acetoacetate. (These pyridine nucleotides must adhere tightly to the islets as they cannot be removed by simple washing.) For the experiments with these islet preparations from six donors, the unstimulated measured acetoacetate levels were subtracted from the glucose-stimu-

lated measured acetoacetate values, and the averages of the individual differences between the stimulated and unstimulated acetoacetate values are shown in the middle of Fig. 9. This average was 3.5–4 times higher in human islets than in rat islets.

In addition, islets from four additional donors were maintained in RPMI 1640 medium containing 5 mM glucose for 24 h before they were washed and incubated in Krebs-Ringer bicarbonate buffer for 30 min. This lowered the background fluorescence to the low values seen with incubating rat islets in the same culture medium prior to the experiment. These experiments shown on the right side of Fig. 9 also showed that glucose stimulated acetoacetate to 3.5–4 times higher levels in human islets than in rodent islets; in addition, in islets from some donors the unstimulated acetoacetate levels were significantly higher than in unstimulated rat islets. Fig. 10 shows that malate, an immediate metabolite of oxaloacetate the product of the PC reaction, was about 50% lower in glucose-stimulated human islets than in similarly stimulated rat islets.

β-Hydroxybutyrate Augments Insulin Release in Human Islets, and the Magnitude of Glucose-stimulated Insulin Release Is Similar to That from Rat Islets—Table 6 shows that β-hydroxybutyrate, the redox partner of acetoacetate, potentiates insulin release from human islets in the presence of a physiological concentration of glucose (5.6 mM) that provides a submaximal stimulus of insulin secretion. Table 6 also shows that human islets stimulated with 5.6 mM glucose or 16.7 mM glucose, which provides a near maximal insulin stimulus, can release the same amount of insulin as rat islets of similar average size and total cellular protein.

DISCUSSION

Pathways That Use Pyruvate Carboxylase and ATP Citrate Lyase or SCOT and Acetoacetyl-CoA Synthetase in Beta Cells—The importance of PC and ATP citrate lyase for insulin secretion in rodent beta cells is underscored by their high levels in rodent islets compared with many other body tissues. As mentioned above, the level of PC in rat islets is known to be relatively high (4, 9–11). In addition, we have found that the activity of ATP citrate lyase in rat islets is >10 times higher than in rat liver and kidney.[3] Also Berne (63) previously reported that the activity of ATP citrate lyase in islets of the New England obese mouse is 4–7 times higher than in liver or kidney. If high levels of PC and ATP citrate lyase are important for insulin release, then it must be explained how human islets with low levels of these two enzymes (Tables 1–3 and Figs. 2–4) achieve the same glucose-stimulated insulin release as rat islets (Table 6) that have high levels of these enzymes.

In the beta cell, as in other cells containing PC, PC can catalyze the unidirectional carboxylation of aerobic-glycolysis-derived pyruvate to form oxaloacetate in the mitochondrial matrix. The oxaloacetate can either be converted to malate, which can be exported from the mitochondria to the cytosol to participate in the pyruvate malate shuttle (3, 4), or oxaloacetate can combine with acetyl-CoA derived from the decarboxylation of pyruvate catalyzed by the pyruvate dehydrogenase complex to form citrate. Citrate, formed by the condensation of oxaloacetate and acetyl-CoA in the citrate synthase reaction, can be exported from the mitochondria to the cytosol where ATP citrate lyase converts the citrate into oxaloacetate and acetyl-CoA. The cytosolic oxaloacetate can be used in the citrate pyruvate shuttle (3, 5, 6), and the acetyl-CoA can be used for short chain acyl-CoA synthesis and lipid synthesis (Fig. 1) (3, 26, 29). However, as we suggest below, there is evidence for another pathway in islets and beta cell lines that can accomplish a similar result in respect to delivering precursors of short chain acyl-CoAs to the cytosol.

Enzyme levels and metabolic and insulin release studies (27–29, 40) suggest an alternative pathway (26) for exporting mitochondrially synthesized products to the cytosol in rodent islets and insulin cell lines and, especially, human islets (this study). In this pathway acetyl-CoA formed in the pyruvate dehydrogenase complex reaction can be converted to acetoacetyl-CoA catalyzed by mitochondrial thiolases (ACAT1 or ACAA2) followed by the reaction of acetoacetyl-CoA plus succinate to form succinyl-CoA plus acetoacetate catalyzed by SCOT. In this scheme, acetoacetyl-CoA and acetoacetate are consumed, and succinate is regenerated in a cycle catalyzed by succinyl-CoA synthetases. The acetoacetate can be exported from the mitochondria to the cytosol where acetoacetyl-CoA synthetase can catalyze the first step in a series of reactions that form short chain acyl-CoAs and lipids in the cytosol (Fig. 1).

Low Pyruvate Carboxylation and ATP Citrate Lyase and a More Active Alternative Pathway Involving Acetoacetate in Human Islets—The "Results" provide evidence to support the idea that the low levels of PC and ATP citrate lyase in human islets compared with rodent islets and clonal rodent beta cell lines are intrinsic properties of human islets and suggest there are important quantitative differences between humans and rodents in mitochondrial biosynthesis and cytosolic pathways used in stimulating insulin secretion. In rodent beta cells, there is no question that PC has a role in insulin secretion. PC has been shown to be responsible for the high rate of carboxylation of pyruvate in rat islets (3, 11, 14–16) and to be involved in cycling of pyruvate in clonal rodent insulin cell lines (17–20), and knockdown of PC inhibits insulin release from clonal insulin cell lines (22) and from rat or mouse islets (23).

The low level of PC in human islets compared with rodent islets and clonal insulinoma cells (Tables 1 and 2 and Figs. 2 and 3) and the low rate of pyruvate carboxylation relative to pyruvate decarboxylation in comparison with rat islets (Table 4) suggest that flux through the PC reaction, although present in the human beta cell, occurs at a lower rate than in rodent beta cells. ATP citrate lyase activity (Table 3) and protein (Fig. 4) were also found to be low in human islets. Might there be pathways of biosynthesis in addition to those involving PC and ATP citrate lyase that are more active in the human beta cell relative to rodent beta cells? In this respect, the level of SCOT in human islets appears to be very high compared with rodent islets (Fig. 6). As described above, SCOT can catalyze the formation of acetoacetate from glucose-derived carbon (Fig. 1), and indeed, acetoacetate increased to 3.5–4-fold higher levels in human islets after 30 min of incubation with glucose than in similarly incubated rat islets (Fig. 9). In contrast, malate, which is an immediate metabolite of oxaloacetate the product of the PC reaction, increased to a lower level in glucose-stimulated

human islets than in glucose-stimulated rat islets (Fig. 10). The lower increase in malate in human islets is also consistent with the lower level of PC in human islets.

Short chain acyl-CoAs by themselves, as well as acting as precursors for long chain acyl-CoAs, have been proposed to have a role in supporting insulin secretion (reviewed in Refs. 3, 64). We recently accumulated a large amount of circumstantial evidence from metabolic and insulin release studies that suggests acetoacetate can act as a carrier of carbon from mitochondria to the cytosol for the synthesis of short chain acyl-CoAs and lipid in beta cells (26–29). Knockdown of either SCOT (65) or acetoacetyl-CoA synthetase (26) in the INS-1 832/13 cell line lowers glucose-stimulated insulin release. In addition, leucine alone, as well as hydroxybutyrate or α-ketoisocaproic acid, which each can be metabolized to acetoacetate, and acetoacetate itself, in combination with other metabolites, stimulate insulin release in INS-1 832/13 cells (27, 28). β-Hydroxybutyrate also potentiates insulin release in the presence of a physiological concentration of glucose in human islets (Table 6) and in rat pancreatic islets as well (27, 66, 67). PC is not directly involved in the metabolism of β-hydroxybutyrate (Fig. 1). Along these lines, it is noteworthy that mitochondria from rat heart, which is a tissue with very low PC (4, 68, 69), when provided with pyruvate, the end product of aerobic glycolysis, synthesize a large amount of acetoacetate (70). It is also notable that in single mouse islets stimulated with glucose and analyzed by capillary LC-MS/MS, the signal for succinate, a substrate of the SCOT reaction, increased 8.3-fold and the signal for acetyl-CoA, a direct precursor of acetoacetyl-CoA, increased 7.7-fold (71). Another fact that supports the idea of an alternate pathway to acetoacetate in addition to a pathway to citrate is that acetoacetyl-CoA, at physiological levels, is a competitive inhibitor of citrate synthase (Fig. 1) in respect to acetyl-CoA, making more acetyl-CoA available for synthesis of acetoacetyl-CoA itself (72). In line with the idea that the human beta cell might preferentially use a pathway in which the SCOT reaction forms acetoacetate for export to the extramitochondrial space where it is converted into acetoacetyl-CoA, which then can be converted to other short chain acyl-CoAs beginning with the acetoacetyl-CoA synthetase reaction (Fig. 1), the level of acetoacetyl-CoA synthetase in human islets is much higher than in rat islets and mouse islets (Fig. 7).

Although there is evidence for metabolic pathways that utilize ATP citrate lyase in rodent islets and rodent insulin cell lines (3, 5, 6), it is noteworthy that the Newgard laboratory studying INS-1 832/13 cells and rat islets (73) and our laboratory studying INS-1 832/13 cells (26) have shown that severely lowering ATP citrate lyase activity with siRNA technology does not inhibit glucose-stimulated insulin release. The Prentki laboratory did achieve inhibition of glucose-stimulated insulin release with knockdown of the enzyme in the INS-1 832/13 cell line (61). The results from the first two studies suggest a pathway redundant with a pathway involving ATP citrate lyase in beta cells.

In contrast to the situation with glucose-stimulated insulin release, BCH-stimulated insulin release was significantly inhibited in our INS-1 832/13-derived cells lines with severely knocked down (>87%) ATP citrate lyase (28). With the very low ATP citrate lyase in these cells, the formation of cytosolic acetyl-CoA from the glutamate-derived citrate to produce other cytosolic short chain acyl-CoAs would be almost nonexistent leaving any short chain acyl-CoA formation in the cytosol to come from acetoacetate exported from the mitochondria (see Fig. 1 to view the acetoacetate and citrate pathways). BCH is a nonmetabolizable leucine analogue that can allosterically activate glutamate dehydrogenase and enhance metabolism of endogenous glutamate to provide carbon to part of the citric acid cycle. Unlike leucine (which also can allosterically activate glutamate dehydrogenase) or glucose, BCH cannot be metabolized to acetyl-CoA and acetoacetyl-CoA to produce acetoacetate. We hypothesized that the inhibited BCH-stimulated insulin release was because short chain acyl-CoA production from glutamate carbon through the acetoacetate pathway was inadequate. Providing a source of acetoacetate by adding β-hydroxybutyrate or α-ketoisocaproate to these ATP citrate lyase-deficient cells raised BCH-stimulated insulin release to that in control INS-1 832/13 cells (28). This experiment also suggests that acetoacetate through the action of acetoacetyl-CoA synthetase can provide the short chain acyl-CoAs required for insulin release.

Conclusions—The pathway that requires SCOT and acetoacetyl-CoA synthetase for the export of acetoacetate from mitochondria to the cytosol to provide substrates for synthesis of short chain acyl-CoAs and lipid in the cytosol (Fig. 1) is present in rat islets and the INS-1 832/13 cell line (26–29). However, this pathway appears to be more active in human islets. It also appears that the pathway that requires PC and ATP citrate lyase is used relatively less (Fig. 1) in human islets than in rodent islets. Glucose acutely stimulates increases in the levels of various lipids in the INS-1 832/13 cell line (29). In addition, glucose carbon (29) and β-hydroxybutyrate carbon (27) are acutely incorporated into lipid in these same cells. In human islets, the level of fatty-acid synthase, which forms longer chain acyl-CoAs, appears to be very high (Fig. 8). The acetoacetate pathway could theoretically very effectively provide short chain acyl-CoAs for the synthesis of cytosolic long chain acyl-CoAs that have long been thought to act as signaling molecules for insulin exocytosis (64). The relative rate of pyruvate carboxylation in human islets, which is 20–30% the relative rate in rat islets (Table 4), should be adequate to support other pathways, including the pyruvate malate shuttle (4) and the pyruvate citrate shuttle (5, 6), which are also probably needed for insulin secretion.

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CASE REPORT

Three Japanese Patients with Beta-Ketothiolase Deficiency Who Share a Mutation, c.431A>C (H144P) in *ACAT1*: Subtle Abnormality in Urinary Organic Acid Analysis and Blood Acylcarnitine Analysis Using Tandem Mass Spectrometry

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Abstract Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency affects both isoleucine catabolism and ketone body metabolism. The disorder is characterized by intermittent ketoacidotic episodes. We report three Japanese patients. One patient (GK69) experienced two ketoacidotic episodes at the age of 9 months and 3 years, and no further episodes until the age of 25 years. She had two uncomplicated pregnancies. GK69 was a compound heterozygote of the c.431A>C (H144P) and c.1168T>C (S390P) mutations in T2 (*ACAT1*) gene. She was not suspected of having T2 deficiency during her childhood, but she was diagnosed as T2 deficient at the age of 25 years by enzyme assay using fibroblasts. The other two patients were identical twin siblings who presented their first ketoacidotic crisis simultaneously at the age of 3 years 4 months. One of them (GK77b) died during the first crisis and the other (GK77) survived. Even during severe crises, C5-OH and C5:1 were within normal ranges in their blood acylcarnitine profiles and trace amounts of tiglylglycine and small amounts of 2-methyl-3-hydroxybutyrate were detected in their urinary organic acid profiles. They were H144P homozygotes. This H144P mutation has retained the highest residual T2 activity in the transient expression analysis of mutant cDNA thus far, while the S390P mutation did not retain any residual T2 activity. The "mild" H144P mutation may result in subtle profiles in blood acylcarnitine and urinary organic acid analyses. T2-deficient patients with "mild" mutations have severe ketoacidotic crises but their chemical phenotypes may be subtle even during acute crises.

Abbreviations

SCOT Succinyl-CoA:3-ketoacid CoA transferase
T2 Mitochondrial acetoacetyl-CoA thiolase

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2, gene symbol ACAT1) deficiency (OMIM 203750) is an autosomal recessive inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. This disorder, first described by Daum et al. (1971), is characterized by intermittent episodes of metabolic ketoacidosis associated with vomiting and unconsciousness often triggered by infections (Fukao et al. 2001). There are no clinical symptoms between episodes. Typical T2 deficiency is easily diagnosed by urinary organic acid analysis, characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate both during ketoacidotic episodes and between episodes (Fukao et al. 2001, 2003). Diagnosis is confirmed by measurement of T2 activity on cultured skin fibroblasts (Robinson et al. 1979; Zhang et al. 2004). T2 deficiency is caused by mutations in the *ACAT1 (T2)* gene located on chromosome 11q22.3-q23.1 (Fukao et al. 1990; Kano et al. 1991). T2 deficiency is very heterogeneous at the genotype level, with at least 50 different mutations described (Fukao et al. 1995, 1997, 1998, 2001, 2002, 2003, 2007, 2008, 2010a, b; Wakazono et al. 1995; Nakamura et al. 2001; Zhang et al. 2004, 2006; Sakurai et al. 2007).

Some T2-deficient patients with mutations which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003). We herein describe three Japanese patients with T2 deficiency whose H144P mutation retains significant residual activity. Their urinary organic acid and blood acylcarnitine profiles were atypical and subtle even during severe ketoacidotic crises.

Materials and Methods

Case Reports

GK69

This Japanese woman (GK69), born in 1984, developed severe metabolic acidosis at the age of 9 months. On admission to a third-level hospital, she was semicomatose, polypneic (48/min), and hypotonic. Laboratory values were: blood glucose 6.8 mmol/L, NH_3 92 μmol/L, blood pH 7.225, pCO_2 7.2 mmHg, bicarbonate 3 mmol/L, base excess −21.3, Na 153 mEq/L (normal range: 139–146), BUN 28.5 mg/dL (normal range: 10–18), and creatinine 1.1 mg/dL (normal range: 0.18–0.46). Metabolic acidosis was refractory to sodium bicarbonate therapy. Peritoneal dialysis was performed for 2 days. On the second hospital day, polypnea and unconsciousness disappeared and the blood gas data improved. Urinary organic acid analysis showed massive amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic aciduria. No increases in 2-methyl-3-hydroxybutyrate or tiglylglycine were noted, although this analysis was performed in an outside laboratory and no urine samples were available for reanalysis. At that time, T2 deficiency was excluded from differential diagnosis based on this organic acid data and the tentative diagnosis was succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. However, an enzyme assay for SCOT was not performed. At the age of 3 years, the patient had a similar but milder episode. Subsequently, she had no further ketoacidotic episodes. Growth and development were normal. She had two uncomplicated pregnancies.

Twin Siblings (GK77b and GK77)

GK77b is a twin Japanese boy. He was born at 36 weeks gestation weighing 2,400 g. His parents had no known consanguinity but both were from a small island in Amami islands in Japan. He experienced several febrile illnesses without ketoacidosis. However, at 3 years 4 months of age, after a 3-day history of fever, cough, and vomiting, he developed anorexia, lethargy, and polypnea. He was admitted to a local hospital. His blood glucose level was 2.3 mmol/L. Blood gas analysis was not performed. Hypoglycemia was corrected with intravenous glucose injection of 20 ml of 20% glucose solution followed by continuous infusion of a 2.6% glucose solution. About 30 h after admission, his condition worsened. Blood gas analysis revealed severe metabolic acidosis showed pH 6.88, pCO_2 6.1 mmHg, and bicarbonate 1.1 mmol/L. He was transferred to a regional hospital. On arrival at the hospital, he was unconscious with a heart rate of 168/min and respiratory rate of 39/min. Blood laboratory data were: WBC 19,050/μL, CRP 0.2 mg/dL (normal values: <0.15), BUN 36.2 mg/dL (normal range: 10–18) creatinine 0.5 mg/dL (normal range: 0.25–0.49), NH3 33.5 μmol/L, glucose 3.8 mmol/L, pH 7.17, pCO_2 20 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 16.3 mmol/L. He received continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.4–0.47 mEq/kg/h. However, unconsciousness and metabolic acidosis did not improve. On the fifth hospital day, he died before being transferred to a third-level hospital.

GK77 is the twin brother of GK77b. Pyloric stenosis was diagnosed at the age of 1 month and corrected surgically;

thereafter, he was well until 3 years 4 months of age. Two days after the onset of his twin brother, he developed frequent repeated vomiting after cough and nasal discharge. Therefore, he was admitted to the regional hospital at the same time as his twin. On admission, he was lethargic. Laboratory findings were: WBC 7,760/μL, CRP 0.5 mg/dL (normal values: <0.15), BUN 20.2 mg/dL (normal range: 10–18), creatinine 0.4 mg/dL (normal range: 0.25–0.49), glucose 3.7 mmol/L, NH3 25 μmol/L, blood pH 7.135, pCO_2 19.5 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 10.1 mmol/L. He received a continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.3 mEq/kg/h. On the third hospital day, his condition worsened and he was transferred to a third-level hospital. On admission, the blood gasses were pH 7.372, pCO_2 21.6 mmHg, bicarbonate 12.2 mmol/L, and base excess −11.2 mmol/L. A glucose infusion rate was further increased to 6.5 mg/kg/min with 10% glucose solution. Acidosis normalized with 9 h (pH 7.399, bicarbonate 21.7 mmol/L, base excess −2.6 mmol/L). Two days later, the urinary ketones became negative and he started eating.

GK77 is now 4 years 8 months and has experienced no further ketoacidotic episodes. The family has been advised to avoid fasting and to come to the local hospital if he has a high fever or appetite loss. His growth and development are within normal ranges.

Urinary Organic Acid Analysis and Acylcarnitine Analysis

Urine samples containing 0.2 mg of creatinine were used for our high risk screening of organic acids. As internal standards, 20 mg each of tropate (TA, C9), margarate (MGA, C17), and tetracosane (C24) were added to these samples. Trimethylsilylated samples were analyzed using capillary gas chromatography-mass spectrometry (QP 5050A, Shimadzu Co. Ltd., Kyoto, Japan), as described earlier (Kimura et al. 1999). The values of organic acids were expressed as the peak area (%) relative to IS-1 (margarate) on the mass chromatogram. Quantification of 2-methyl-3-hydroxybutyrate and tiglylglycine in urine samples from GK77b and GK77 was kindly done by Dr. Sass (Freiburg University) (Lehnert 1994). For comparison, quantification was also done in urine samples from T2-deficient patients whose urinary screening profiles had typical T2 deficient ones. We used urine sample in stable condition from GK01 who is a compound heterozygote of A333P and c.149delC (Fukao et al. 1998) and samples in acute and stable conditions from T2-deficient patients from India (GK(Ind)) in our high-risk screening. Blood spot and serum acylcarnitine analysis using tandem mass spectrometry was also done, as described (Kobayashi et al. 2007), and blood spot samples from GK75 and GK79, who are R208X homozygotes (Fukao et al. 2010b) were used as positive controls.

Enzyme Assay and Immunoblot Analysis Using Fibroblasts

Control and patients' fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described (Robinson et al. 1979; Zhang et al. 2004). We assayed acetoacetyl-CoA thiolase activity in the presence and absence of potassium-ion, since T2 is the only thiolase which is activated by the ion. Immunoblot analysis was done, as described (Fukao et al. 1997). In the cases of the controls, twofold serial dilution samples from 30 to 3.75 μg were electrophoresed together with samples (30 μg) of GK68 and GK77 to determine the amount of T2 protein in the patients' fibroblasts relative to that in the control fibroblasts.

Mutation Analysis

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Genomic DNA was extracted from fibroblasts using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed by PCR and direct sequencing of genomic fragments that included each exon and its surrounding intron sequences (Fukao et al. 1998). For GK77b and the parents, exon 5 was amplified from a dried blood spot 1.25 mm in diameter, which was used for tandem mass spectrometry, using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

Restriction Enzyme Assay to Detect c.431A>C (H144P)

The c.431A>C (H144P) mutation creates a new BmgT120 I site (GGACC). DNAs from 110 Japanese controls were examined using a restriction enzyme assay, as follows.

A fragment (314 bp), including exon 5 and its surrounding introns, was amplified using the following primers:

In4 as (in intron, −69 to −48)5′-CATGCTCTATTAAG-TTCTGCAG-3′

In5 as (in intron, +137 to +119) 5′-ATCCAGACACTCT-TGAGCA-3′

An aliquot of the resulting amplicon was digested with BmgT120 I, then resolved on a 5% polyacrylamide gel. The c.431A fragment (wild-type) is 314-bp long and the c.421C fragment is cut into 162-bp and 152-bp fragments.

Transient Expression Analysis of Mutant cDNAs

Transient expression of T2 cDNAs was performed using a pCAGGS eukaryote expression vector (Niwa et al. 1991), as described (Sakurai et al. 2007). After transfection, cells were cultured at 37°C or 40°C for 48 h, then harvested and kept at −80°C until use. Cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100. After centrifugation at 10,000 × g for 10 min, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity and for immunoblot analysis.

Results and Discussion

Confirmation of the Diagnosis

GK69's fibroblasts were assayed for SCOT activity to confirm the diagnosis in 2008, when GK69 was 24 years old. As shown in Table 1, she was diagnosed as having T2 deficiency but not as having SCOT deficiency.

SCOT deficiency was first suspected in GK77 and GK77b, based on the following facts (1) Two of the four SCOT deficient Japanese families were from the Amami islands, the population of which is about 120,000. They were T435N homozygotes (Fukao et al. 2004). (2) The acylcarnitine profiles and urinary organic acid analysis during acute ketoacidotic crisis in both patients had no typical profile for T2 deficiency, as discussed below. As shown in Table 1, GK69's and GK77's fibroblasts had normal SCOT activity and a higher ratio (1.3) of acetoacetyl-CoA thiolase activity in the presence to the absence of potassium ions than typical T2-deficient fibroblasts (the ratio was around 1.0). Immunoblot analysis also showed a clearly detectable amount of T2 protein in GK77's fibroblasts, and a lower amount in GK69's fibroblasts. Densitometric analysis showed that the amounts of T2 protein in GK77 and GK69 were estimated to be 50% and 25% of control, respectively (Fig. 1).

Table 1 Acetoacetyl-CoA thiolase activities in the absence and presence of potassium ions

Fibroblasts	Acetoacetyl-CoA thiolase activity			SCOT activity
	$-K^+$	$+K^+$	$+K^+/-K^+$	
Controls ($n = 5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1
GK69	3.6 ± 0.5	4.1 ± 0.9	1.2 ± 0.1	4.7 ± 1.4
GK77	4.2 ± 0.3	5.8 ± 1.5	1.4 ± 0.3	3.9 ± 0.5
T2D	4.5 ± 1.4	4.7 ± 1.6	1.0 ± 0.1	5.6 ± 0.5

Enzyme activity is expressed as nmol/min/mg of protein. In cases of patients, enzyme assay was done three times and shows average ± SD. T2D, A disease control

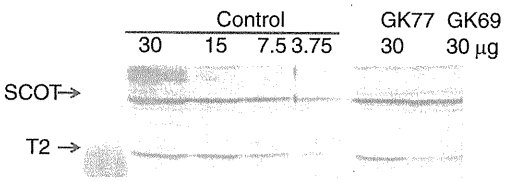

Fig. 1 Immunoblot analysis. In the cases of the controls, serial twofold dilutions from 30 to 3.75 μg were studied together with samples (30 μg) from GK68 and GK77. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

Mutations and Their Effects on T2 Protein

Mutation screening revealed that GK69 was a compound heterozygote of c.431A>C (H144P) and c.1168T>C (S390P). Her mother had S390P heterozygously but did not have H144P. The father's DNA was not available for analysis. GK77 had an H144P mutation homozygously, shown by mutation screening at the genomic level. Their parents and GK77b were heterozygous carriers and a homozygote of H144P, respectively. The c.431A>C (H144P) mutation creates a BmgT120I site (GGACA to GGACC). We could not find c.431A>C (H144P) in the 110 Japanese controls using the restriction enzyme assay with BmgT120I.

We performed transient expression analysis of wild-type and mutant cDNAs in T2-deficient SV40-transformed fibroblasts. Following expression of T2 cDNAs for 48 h at 37°C, an enzyme assay and immunoblots were performed (Fig. 2a,b). The transfection of wild-type T2 cDNA produced high potassium ion-activated acetoacetyl-CoA thiolase activity (T2 activity), whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. The H144P mutant retained a residual T2 activity of ~25% of the wild-type value (Fig. 2a). The S390P mutant did not retain any residual T2 activity. In immunoblot analysis (Fig. 2b), the H144P mutant protein was detected, whereas no S390P protein was detected. The relative amount of the H144P mutant protein, as compared to the wild-type, was estimated to be 50%. Hence, the specific activity (unit/mg of T2 protein) of the H144P mutant protein was estimated to be about 50% of the wild type. Protein-folding and post-folding stability is predicted to vary with the incubation temperature. Hence, we also performed transient expression at 40°C for 48 h. The H144P mutant in expression at 40°C had a similar level of residual activity to that at 37°C.

We reported the tertiary structure of the human T2 tetramer (Haapalainen et al. 2007). Figure 3a shows the positions of the H144P and S390P mutations on the dimer.

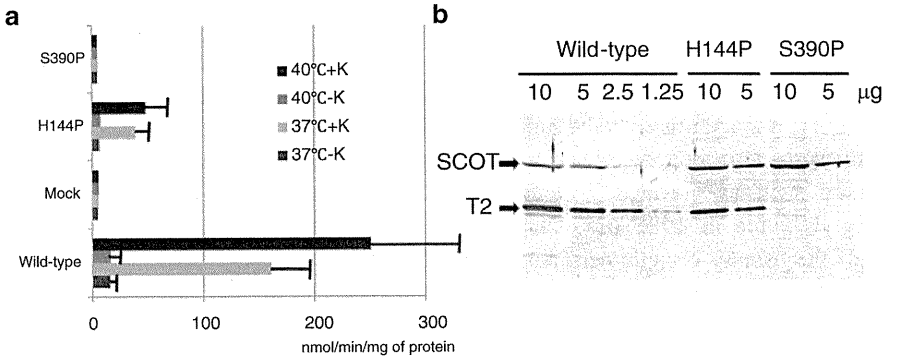

Fig. 2 Transient expression analysis of H144P and S390P mutant cDNAs. Transient expression analysis was performed at 40°C and 37°C. (**a**) Potassium ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence (−K) and presence (+K) of potassium ions are shown together with the SD of three independent experiments. (**b**) Immunoblot analysis. The protein amounts applied are indicated above the lanes. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

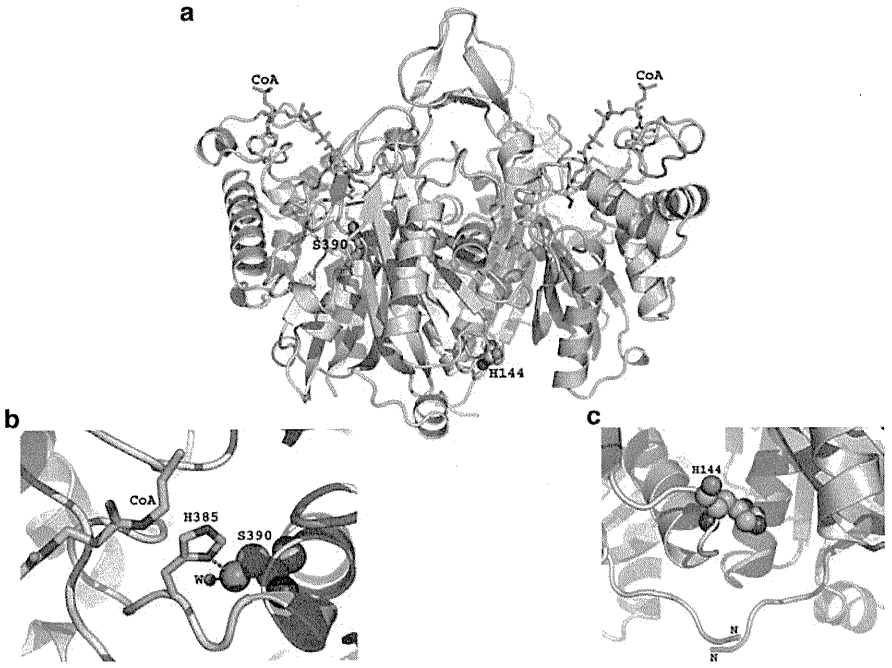

Fig. 3 The positions of H144P and S390P on the tertiary structure of human T2 dimers with substrates of coenzyme A

As seen in the figure, S390 is close to the active site and H144 is at the dimer interface close to the surface of the protein. Figure 3b shows a zoomed-in view around S390. This mutant is located at the active site. S390 is hydrogen-bonded to catalytic histidine, H385; it could be that this serine is needed to orient histidine in a way that the histidine can stabilize the transient negative charge of the substrate optimally. S390 is also hydrogen-bonded to a water molecule that is needed in stabilizing parts of the enzyme. So, if S390 is mutated into proline, these two hydrogen bonds do not exist. Hence, this S390P is expected to bring about a serious change in T2 catalytic cavity. In our expression analysis, this S390P was also too unstable to detect mutant protein. Figure 3c shows a zoomed-in view at the dimer interface. H144 is interacting with the residues of the neighboring subunit. If this residue is mutated into Pro, there is less dimeric interaction, which in turn might destabilize the overall structure. Since this residue is far from the active site and substrate binding site, it is difficult to explain why this H144P mutant had reduced specific activity in transient expression analysis from the viewpoint of structural analysis.

Urinary Organic Acid Analysis

GK69 was first suspected to having T2 deficiency as a probable diagnosis; however, urinary organic acid analysis at the first ketoacidotic crisis indicated no characteristic profile for T2 deficiency such as elevated 2-methyl-3-hydroxybutyrate and tiglylglycine in 1985 (no data was available). The results of the urinary organic acid analysis of our patients are shown in comparison with those of typical T2-deficient patients, GK01 and GK(Ind) (Table 2, Fig. 4). At the age of 24 years when her condition was stable, GK69's urinary organic acid analysis showed that there were only trace amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine (Table 2). In our screening, this low level of tiglylglycine was difficult to detect. Urinary organic acid analysis during the acute crises of GK77 and GK77b showed huge amounts of 3-hydroxybutyrate and acetoacetate with elevated 2-methyl-3-hydroxybutyrate but only trace amounts of tiglylglycine. The levels of 2-methyl-3-hydroxybutyrate and tiglylglycine during a stable condition in GK77 are similar with those in GK69.

In cases of typical T2-deficient patients, it is easy to suspect T2 deficiency based on large amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine as shown in Fig. 4. However, even in cases of trace amounts of tiglylglycine (possibly under the detection limit), T2 deficiency cannot be excluded. An H144P mutation, which retained high residual activity, may contribute to atypical profiles in the presented cases. These findings strengthen our previous observations that some T2-deficient patients with mutations, which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003).

Table 2 Quantitative analysis of urinary organic acid analysis during acute crises and stable conditions

Patients	Acute crises		Stable conditions	
	2M3HB	Tiglylglycine	2M3HB	Tiglylglycine
GK69	NA	NA	14.0	13.3
GK77b	405.7	45.8	NA	NA
GK77	160.2	6.7	27.3	14.8
GK01	NA	NA	399.1	732.1
GK(Ind)	484.6	503.9	195.1	797.6
Controls ($n = 42$)			10.7 ± 7.6	24.6 ± 14.6

Values are expressed as mmol/mol creatinine

NA means that samples were not available for the analysis. GK01 is a compound heterozygote of c.149delC and A333P, which retained no residual activity (Fukao et al. 1998). GK(Ind) indicates a patient with typical T2-deficient profiles of urinary organic acids in our screening

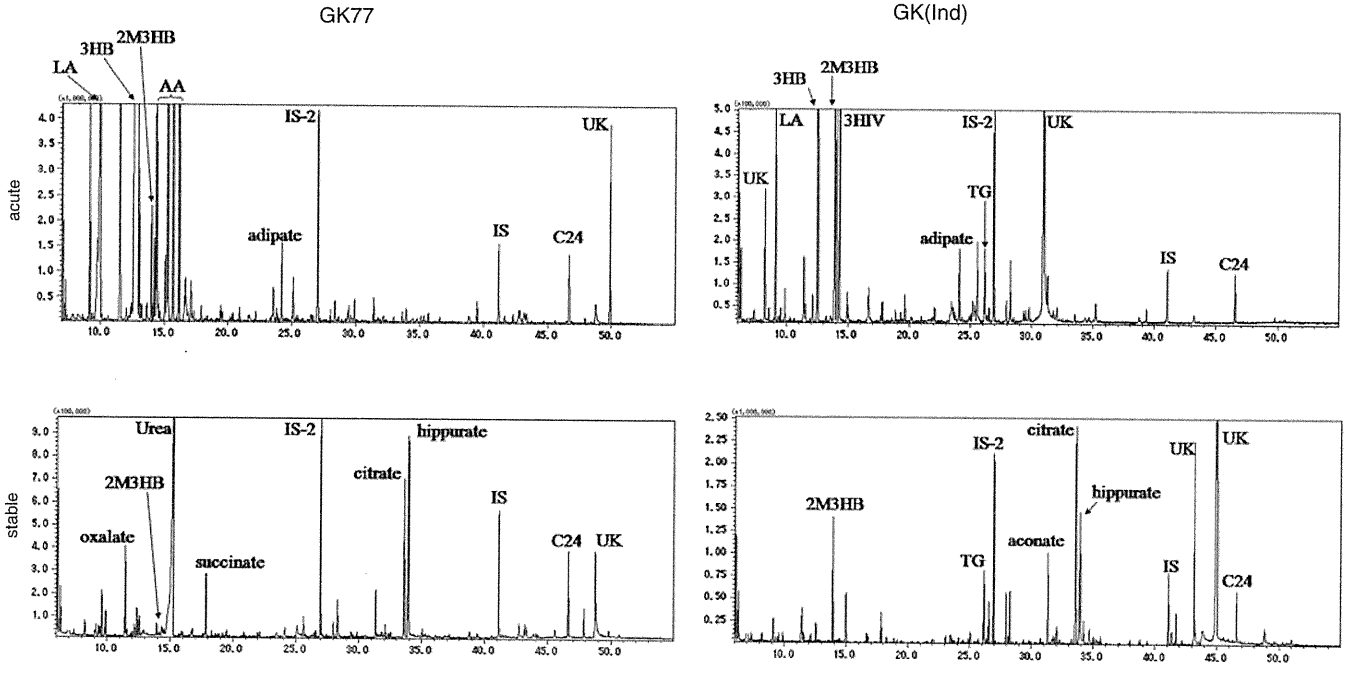

Fig. 4 Urinary organic acid profiles of GK77 during the acute episode and an asymptomatic period in comparison with those of a typical T2-deficient patient (GK(Ind)). *LA* Lactate, *3HB* 3-OH-butyrate, *3HIV* 3-OH-isovalerate, *AA* Acetoacetate, *2M3HB* 2-Methyl-3-OH-butyrate, *TG* Tiglylglycine, *IS-2* and *IS* Internal standards, *UK* Unknown. Since acetoacetate is unstable and samples from GK(Ind) were shipped on filter papers after thoroughly drying, the levels of acetoacetate are likely underestimated

Table 3 C5-OH and C5:1 carnitines in blood filters and serum samples from GK77 and GK77b during acute crises

Patients	Dried blood spots		Serum	
	C5:1	C5-OH	C5:1	C5-OH
GK77b	0.027	0.11	ND	0.12
GK77	0.012	0.11	0.044	0.10
R208X homozygotes				
GK75 (acute)	0.89	2.89	NA	NA
GK79 (stable)	1.20	2.35	NA	NA
Controls ($n = 30$)				
Average ± SD	0.015 ± 0.016	0.26 ± 0.15	0.015 ± 0.013	0.059 ± 0.024

ND not detected, *NA* not applicable

The values are expressed as μmol/L

GK75 and GK79 are positive controls for T2 deficient patients who are R208X homozygotes (Fukao et al. 2010b)

Blood and Serum Acylcarnitine Analyses

Acylcarnitine analysis was done using samples during the acute crises of GK77 and GK77b. Table 3 shows the results in comparison with those of typical T2-deficient patients (R208X homozygotes) (Fukao et al. 2010b). C5:1 and C5OH elevation in blood spots, characteristic for T2 deficiency, was clearly detected in the samples from the typical T2-deficient patients but was absent in samples from GK77 and GK77b. We previously reported that the abnormality of the acylcarnitine profiles in T2-deficient patients with mutations which retain some residual activity is subtle during nonepisodic conditions (Fukao et al. 2003), but the present study clearly showed that it could be also subtle even during severe ketoacidotic episodes. This means that acylcarnitine analysis using blood spots cannot detect some T2-deficient patients like GK77 and GK77b. Serum acylcarnitine analysis might detect elevation of these compounds to some extent, but we need to analyze more cases to clarify the usefulness of serum acylcarnitine analysis in such T2-deficient patients with mutations which retain some residual activity.

T2 deficiency cannot be excluded even if acylcarnitine profiles during acute episodes are within normal ranges. Careful evaluation of urinary organic acids, especially for the presence of 2-methyl-3-hydroxybutyrate, is necessary not to overlook T2 deficiency.

Clinical Issues

Since they were confirmed as identical twins by DNA analysis (data not shown), their genetic backgrounds were identical and most environmental factors were also very similar between them. One died during the ketoacidotic crisis and the other survived.

In Japan, intravenous infusion therapy for vomiting, appetite loss, and dehydration is commonly performed with commercially available initial infusion solution, such as Solita T1 (2.6% glucose) followed by maintenance solution, such as Solita T2 and T3 (4.3% glucose). These solutions are effective for physiological ketosis. However, in the case of T2 deficiency, a higher concentration of glucose may be necessary. Accordingly, we had the impression that GK77 became much better after the glucose concentration was changed from 5% to 10%. In the case of prolonged ketoacidosis, consideration should be given to increasing the infusion rate of glucose to ensure high normal blood glucose level to suppress ketone body synthesis and isoleucine catabolism via insulin secretion.

Acknowledgments We thank professor Jörn Oliver Sass (Freiburg Univ) for quantification of urinary 2-methyl-3-hydroxybutyrate and tiglylglycine, Drs Hironori Kobayashi and Yuichi Mushimoto (Shimane University) for urinary organic acid analysis and tandem mass analysis, Dr Tamayo Ishikawa (Kagoshima University) for patients' care, and Ms Keiko Murase and Ms Naomi Sakaguchi (Gifu University) for technical assistance. We also thank Paul Langman, PhD for his assistance with scientific English usage.

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Concise One-Sentence Take-Home Message

Patients with beta-ketothiolase deficiency having a mutation which retains some residual activity showed subtle abnormality in urinary organic acid analysis and blood acylcarnitine analysis even during acute ketoacidotic episodes.

Details of the Contributions of Individual Authors

Toshiyuki Fukao and Naomi Kondo performed the enzyme assays, immunoblot/mutation analysis, and expression analysis of cDNAs. Toshiyuki Fukao mainly wrote this manuscript. Shinsuke Maruyama, Toshihiro Ohura, Mitsuo Toyoshima, Naomi Kuwada, and Mari Imamura are the physicians responsible for the patients. Yuki Hasegawa and Seiji Yamaguchi performed gas chromatography-mass spectrometry and tandem mass spectrometry analyses and first suspected the disorder. Isao Yuasa confirmed GK77 and 77b as identical twins by DNA analyses. Antti M Haapalainen and Rik K Wierenga analyzed the tertiary structural effects of the mutations.

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Mitochondrial acetoacetyl-CoA thiolase, acetyl-CoA acetyltransferase 1 (EC 2.3.1.9)

ACAT1 gene (gene ID 38, NM_000019.3)

Details of Funding

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Details of Ethics Approval

This study has been approved by the Ethical Committee of the Graduate School of Medicine, Gifu University.

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原　著

カルニチンパルミトイルトランスフェラーゼ2欠損症のろ紙血血清のアシルカルニチンプロファイルの経時的変化

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要　旨

我々は，カルニチンパルミトイルトランスフェラーゼ(CPT)2欠損症の血液ろ紙，血清のアシルカルニチンプロファイルの出生後からの経時的変化を検討した．症例は日齢0の男児．切迫早産のため在胎37週0日，帝王切開にて出生した．姉がCPT2欠損症のため本症例もブドウ糖輸液を行い注意深い観察を行った．血液ろ紙，血清のアシルカルニチンプロファイルを経時的に分析し，以下の所見と姉がCPT2欠損症と酵素診断されていることから本症例は無症状であったがCPT2欠損症と化学診断した．血液ろ紙におけるC16-アシルカルニチン(C16)，C18：1アシルカルニチン（C18：1），C18-アシルカルニチン（C18）は日齢3にピークとなり，カットオフ値を超えていたがその後カットオフ値以下となった．(C16＋C18：1)/C2は生後14日までカットオフ値を超えており，スクリーニング指標として有用と考えられた．血清でもC16, C18：1, C18は日齢3にピークとなり，その後徐々に低下したが，日齢14まで常にカットオフ値を超えており，ろ紙血よりも血清におけるアシルカルニチン分析の方が確実に異常を指摘できた．ろ紙血による現行の採血時期における脂肪酸代謝異常症のスクリーニングでは，我々の症例のようにすでにC16, C18, C18：1がカットオフ値を下回り偽陰性となる可能性がある．このようなCPT2欠損症例を見逃さないためにはスクリーニング時期をより早期に設定する必要性が示唆された．

キーワード：CPT2欠損症，脂肪酸β酸化障害，アシルカルニチン，
タンデムマススペクトロメトリー，新生児マススクリーニング

はじめに

カルニチンパルミトイルトランスフェラーゼ(CPT)2欠損症は，常染色体劣性遺伝形式を示し，ミトコンドリア脂肪酸β酸化障害をきたす疾患の1つである．ミトコンドリアにおける脂肪酸β酸化系は肝臓ではブドウ糖からのエネルギー供給が低下したときなどに作動してアセチル-CoAやケトン体など代替エネルギーを産生する．また脂肪酸β酸化系は心臓や骨格筋においては安静時のエネルギー産生において重要である．長鎖脂肪酸が細胞質からミトコンドリア内に輸送される際にカルニチンシャトルが必要である．長鎖脂肪酸が活性化されたアシル-CoAはミトコンドリア外膜に存在するCPT1により，アシルカルニチンとなる．アシルカルニチンはカルニチンアシルカルニチントランスロカーゼによりミトコンドリア内膜を通過し，CPT2により再びアシル-CoAへ変換される．CPT2に異常があるとミトコンドリア内でアシルカルニチンからアシル-CoAへの変換が障害され，β酸化を受けることができず，アシルカルニチンが蓄積する．このように脂肪酸代謝が十分に行われず，エネルギー産生が低下することで発症する．

CPT2欠損症は本邦におけるタンデム型質量分析計(以下タンデムマス)によるマススクリーニング・パイロット研究などの報告によれば，比較的頻度の高い脂肪酸酸化異常症である[1]．

臨床型は大きく出生前発症型，乳幼児発症型，軽症型（骨格筋型）の3つに分類される．出生前発症型は腎異形性，大脳奇形，顔貌異常など認め，致死的である．乳児発症型は低ケトン性低血糖の発作として発症し，乳幼児突然死やReye様症候群と関連がある．軽症型（骨格筋型）は成人期に偶発性横紋筋融解症で発症する[2]．

我々は以前1歳3か月にReye様症候群で発症したCPT2欠損症症例を経験した[3]．今回その次子で，出生

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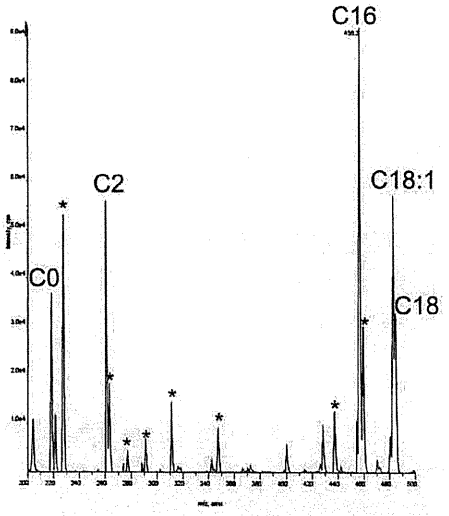

C0 14.16 (下限カットオフ値10.0)
C2 9.7 (下限カットオフ値5.0)

C16 6.64 (上限カットオフ値7.0)
C18:1 2.64 (上限カットオフ値3.2)
C18 2.08 (上限カットオフ値3.2)

C18:1＋C16／C2 0.96
(上限カットオフ値0.35)

図1 生後12時間後のろ紙血アシルカルニチンプロファイル
横軸はm/z値，縦軸は相対量を示した．図右に各アシルカルニチンの定量値（μmol/l）と括弧内に上限または下限カットオフ値を示した．＊は内部標準物質．C0，C2に比較してC16，C18：1，C18などの長鎖アシルカルニチンが高く，(C18：1＋C16)/C2も上昇しており，CPT2欠損症に特徴的なパターンである．

直後から経過観察し，生後12時間後から経時的に血清，ろ紙血のアシルカルニチン推移を観察し，CPT2欠損症と化学診断できた症例を経験した．タンデムマスによる新生児スクリーニングの実施時期を考える上で貴重な経験であると考え報告する．

症　例

在胎週数37週0日，出生体重2,600g，男児．

家族歴：姉がCPT2欠損症で当科にて加療中．姉は1歳3か月にReye様症候群にて発症した．発作時の有機酸分析にて低ケトン性ジカルボン酸尿，アシルカルニチン分析にてCPT2欠損症が疑われた．線維芽細胞を用いたCPT2活性がコントロールの16％と低下しておりCPT2欠損症と診断した．ゲノムレベルでの遺伝子解析では父由来の*CPT2*遺伝子にE174K変異が同定されたが，母由来の変異は同定されなかった[3]．

母親の妊娠経過：次子妊娠にあたり，遺伝相談を実施した．姉で母由来の変異は同定されておらず，出生前に遺伝子解析を行っても保因者か患者かの区別がつけられないこと，新生児期に十分なグルコースの補給で新生児期発症を予防できる可能性が高いことを説明し，両親の希望で出生前検査は行わずに妊娠は継続された．妊娠36週6日，切迫早産にて入院．翌日緊急帝王切開となった．

出生後の経過：アプガースコア1分9点，5分10点で仮死なく出生．体温36.8℃，呼吸数54回/分，心拍数134回/分，血圧59/30mmHgで活気は良好であった．大泉門は平坦，肺野は清，心音は整，腹部は平坦で軟，筋トーヌス低下や外表奇形を認めなかった．血液生化学検査では，アンモニア値は出生後157μg/dlとやや高値であったが，生後3日には100μg/dl以下となり一過性であった．血糖値は46mg/dlと著明な低血糖（40mg/dl以下）は認めず，その後も低血糖は認めなかった．その他，胸部レントゲンではCTR47％で心拡大はなく，心臓，腎，頭部超音波検査では異常を認めなかった．

出生後10％グルコースにてグルコース注入速度(GIR)4.8mg/kg/minの糖補充を開始した．両親の承諾のもと出生後早期に遺伝子解析を実施したところ患児もE174K変異をヘテロでもつことが判明し，注意深い観察をおこなった．日齢1に10ml×8回/日から経管栄養を開始し，以後1回哺乳量を10mlずつ増量し，輸液は漸減していった．日齢5に経静脈栄養を中止し自律哺乳とした．

方　法

日齢1，2，3，4，5，6，7，8，14にろ紙血，血清を採取し，タンデムマスによるアシルカルニチン分析を島根大学において，既報の方法にて行った[4]．

結　果

生後12時間（日齢1）での血液ろ紙のアシルカルニチンの結果は，C16は6.64μmol/L，C18：1は2.64μmol/L，C18は2.08μmol/Lと長鎖アシルカルニチンが高値であったがカットオフ値以下であった．しかし，(C18：1＋C16)/C2は0.96とカットオフ値を超えて高

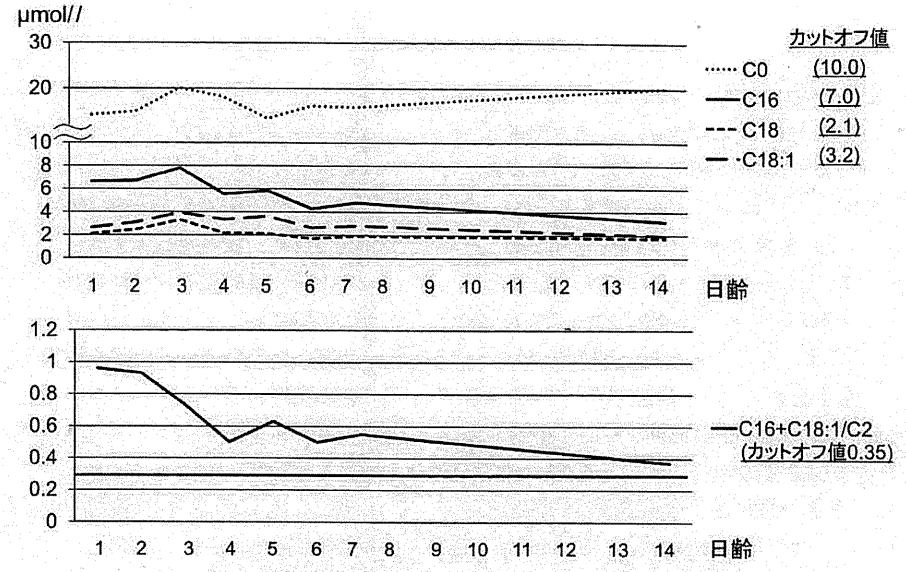

図2　血液ろ紙アシルカルニチンプロファイルの推移
上段の図では横軸は日齢，縦軸は各アシルカルニチン量．括弧内に上限または下限カットオフ値を示した．C16，C18，C18：1はいずれも日齢3にピークとなり，その後漸減した．遊離カルニチンは明らかな低値を認めない．下段の図は（C16＋C18：1）/C2を縦軸に示した．図中にカットオフ値を直線で示した．出生直後が最も高く，日齢14まで上限値を超えている．

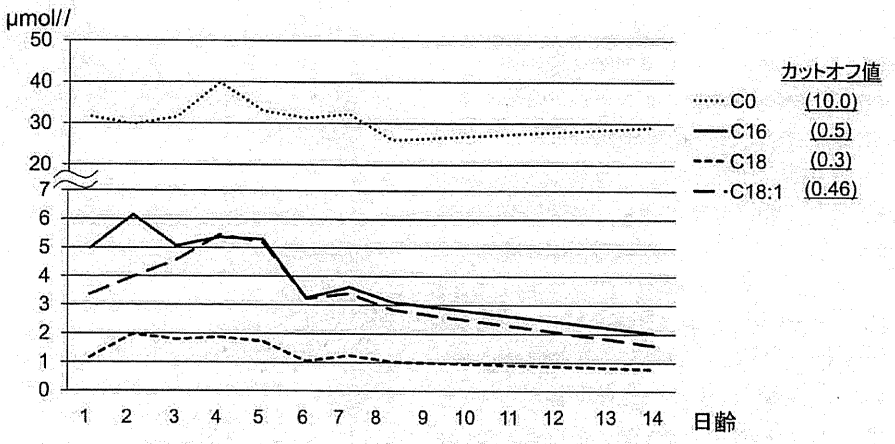

図3　血清アシルカルニチンプロファイルの推移
横軸は日齢，縦軸は各アシルカルニチン量．括弧内に上限または下限カットオフ値を示した．日齢1より明らかな長鎖アシルカルニチンの増加を認める．C16，C18，C18：1は日齢4にピークとなりその後低下した．遊離カルニチンは明らかな低値を認めない．

値[1]であった（図1）．

血液ろ紙アシルカルニチンプロファイルの推移を示す（図2）．C16, C18, C18：1はいずれも日齢3にピークとなり，その後漸減した．遊離カルニチンは日齢3に20.1μmol/Lまで上昇した後，若干の低下傾向を示したが，カットオフ値以下にはならなかった．CPT2欠損症のスクリーニング指標である（C16＋C18：1）/C2の値の変化は，特に出生直後が最も高い結果となっており，以後徐々に低下しているが，日齢14まで上限値を超えていた．

次に血清カルニチンのプロファイルを示す（図3）．日齢1の結果にて，C16は4.97μmol/L，C18：1は3.36μmol/L，C18は1.16μmol/Lと長鎖アシルカルニチンの増加が認められ，姉がCPT2欠損症と酵素診断されていることを考えてCPT2欠損症と化学診断した．血清C16, C18, C18：1は日齢4にピークとなりその後低下した．血清中遊離カルニチンは日齢4の39.87μmol/Lをピークに低下傾向となった．

考　察

CPT2欠損症では，血清中アシルカルニチン分画においてC2の低下，C16, C18, C18：1などの長鎖アシルカルニチンの上昇や(C16+C18：1)/C2の上昇をスクリーニング指標にして精査，診断に結びつける．

本症例における血液ろ紙ではC16, C18, C18：1の長鎖アシルカルニチンは日齢3にピークとなったが，上限カットオフ値をやや超える程度であった．しかし，血清中のC16, C18, C18：1は少なくとも日齢14まではカットオフ値を超えており，血清でのアシルカルニチン分析のほうが血液ろ紙に比較してより確実に異常を指摘できることが分かった．(C16+C18：1)/C2の値に関しては，血液ろ紙においては日齢14までカットオフ値を超えていた．血液ろ紙でスクリーニングを行う場合を行う場合，(C16+C18：1)/C2をより重視すべきであると考えられた．

遊離カルニチンは新生児期早期には低下を認めなかった．しかし，遊離カルニチンの低値を伴う二次性カルニチン欠乏が乳幼児発症型CPT2欠損症の患児でみられる[2)5)]ため今後注意が必要である．患児の姉も発症時に遊離カルニチンの著明な低値を認めていた．

カルニチン欠乏がみられた場合はL-カルニチンの補充が重要である．また，カルニチン欠乏が明らかになる前に予防的な投与を考慮してもよいと思われる．

CPT2欠損症を含む先天代謝異常症のタンデムマススクリーニングは欧米をはじめとして各国で新生児マススクリーニングに導入されており，脂肪酸酸化異常症の早期発見に寄与している．血液ろ紙による新生児マススクリーニングの施行時期は，アメリカでは日齢1～2[6)]に行われ，本邦におけるパイロットテストには日齢4～6のろ紙血が利用されている．本症例の結果では，血液ろ紙では長鎖アシルカルニチンのピークが日齢1～3にあり，その後減少していた．このことから日齢4～6に採取したろ紙血によるタンデムマススクリーニングでは，すでに長鎖アシルカルニチンは低下し始めており偽陰性となる可能性がある．そのため，本邦におけるスクリーニング採血時期を海外と同様にさらに早い時期に行う必要性があるのではないかと考えられる．一方で血清アシルカルニチンではいずれの時期でもカットオフ値を超えていた．血液ろ紙分析でカットオフ値を超えていたC14：1アシルカルニチンが経過観察中にカットオフ値を下回った極長鎖アシル-CoA脱水素酵素欠損症症例が報告されており[7)]，スクリーニングの再検査や経過追跡には血清アシルカルニチン分析を行うことがよいと考えられる．

また，本症例においては出生時にCPT2欠損症を疑わせるような症状は認めなかったが，生後12時間後の検体からすでにCPT2欠損症を示唆するアシルカルニチンプロファイルであった．特に本患者で同定されている*CPT2*遺伝子のE174K変異は，日本人成人型で同定された変異で，10%程度の残存活性を持っている変異である[8)]．母由来の変異は同定されていないが，少なくともCPT2の残存活性をもつために姉は新生児型でなく，乳幼児期発症型になったと考えられる．このような残存活性を持つ症例において，さらに持続的に糖補充をしていたにもかかわらず生後12時間からすでに血液ろ紙，血清のいずれにおいても異常が指摘された．

新生児早期からタンデムマス解析で異常を指摘できることから，新生児タンデムマススクリーニングの普及により，このような症例の発症前診断が可能となり，早期の治療的介入，指導により，発症の回避が可能になると思われた．

結　語

CPT2欠損症の血液ろ紙，血清のアシルカルニチンプロファイルの経時的変化を観察した．生後12時間後の検体からすでにCPT2欠損症を示唆するアシルカルニチンプロファイルであった．ろ紙による現行の採血時期におけるスクリーニングでは，すでにカットオフ値を下回っている可能性があり，スクリーニング時期をより早期に設定する必要性があるのではないかと考えられた．

日本小児科学会の定める利益相反に関する開示事項はありません．

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Carnitine Palmitoyltransferase-2 (CPT2) Deficiency：Time-dependent Changes of Acylcarnitine Profiles in Dried Blood Spots and Serum after Birth

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We analyzed time-dependent changes of acylcarnitine profiles in dried blood spots and serum samples after birth in a CPT2-deficient patient. The boy was born at 37 weeks gestation via Caesarean section. Since his sister had CPT2 deficiency, he was carefully followed with intravenous glucose infusion from birth to day 6. Although he had no clinical symptoms, he was also diagnosed as CPT deficiency based on the family history and acylcarnitine analyses. In the acylcarnitine analyses using dried blood spots, peak levels of C16, C18, and C18：1 acylcarnitines, which are the usual screening markers for CPT2 deficiency, were above their upper cutoff values on day 3. However, their levels decreased and were under the cutoff values thereafter. The ratio C16＋C18：1/C2 was above the upper cutoff values until day 14, indicating that the ratio is a useful screening marker for CPT2 deficiency. In contrast, for acylcarnitine analyses using serum, although the peak levels of C16, C18, and C18：1 acylcarnitines were also detected on day 3, their levels declined gradually but still were above their upper cutoff values until day 14. These facts indicate that acylcarnitine analyses using serum detected this abnormality more effectively than using dried blood spots. Therefore, screening for fatty acid oxidation using dried blood spots on day 5 may result in a false-negative result since the values of C16, C18, and C18：1 acylcarnitines were under their cutoff values in our CPT2 deficient patient. Screening earlier than on day 5 may be considered to detect CPT2-deficient patients like this case.