Table 5
Analytical results obtained for blood spot samples from 5 patients.

Disease		Val		Leu		Ile		Leu + Ile		Met		Phe	
		[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]	[μmol/l]	Ratio[e]
PKU[a]	1	110.7	0.40	69.8	0.34	40.6	0.36	110.3	0.36	9.4	0.17	***619.0***	***6.01***
	2	*189.3*	0.68	124.3	0.61	66.5	0;90	190.8	0.62	8.7	0.16	***201.6***	***1.96***
MSUD[b]	1	***360.3***	***1.29***	***2646.6***	***13.07***	***141.4***	***1.27***	***2788.0***	***9.08***	4.6	0.08	40.4	*0.39*
	2	***297.0***	***1.06***	***1017.4***	***5.02***	***257.0***	***2.30***	***1274.4***	***4.15***	6.6	0.12	43.7	*0.42*
NICDD[c]	1	178.1	0.64	91.6	0.45	49.9	0.45	141.5	0.46	***300.8***	***5.39***	***168.0***	***1.63***
Control[d]													
Mean		166.0	0.59	105.5	0.52	59.9	0.54	165.3	0.54	25.9	0.46	60.3	0.59
Cut-off		279.7	1.00	202.5	1.00	111.5	1.00	307.0	1.00	55.8	1.00	102.9	1.00

[a] Phenylketonuria.
[b] Maple syrup urine disease.
[c] Hypermethionine and neonatal intrahepatic cholestasis caused by citrin deficiency.
[d] 33 healthy controls.
[e] Ratio to cut-off value.

From these results, total analysis time was 80 min, which included a 60-min extraction time, a 10-min purification and derivatization time and a 10-min fast-GC/MS analysis time that included column cool-down. Although the extraction time was relatively long, it could be easily shortened by processing more samples in a batch. If 10 samples were processed as one batch, the analysis time for each sample would be only 6 min extraction time. In the reported results, total analysis time per sample with this method was 26 min and could be dramatically shortened compared to the conventional method.

The evaluated method was applied to amino acid analysis in a blood spot punch, and the method detection limits were determined (Table 3). The MDLs of Val, Leu, Ile, Leu + Ile, Met and Phe were lowered by factors of 5.96, 8.23, 14.95, 5.42, 5.97 and 16.20, respectively, compared with cut-off values (Table 5). The maximum concentrations were up to 11.56, 12.13, 12.82, 12.33, 37.2 and 7.98 times higher compared to cut-off values. These results show that this method can be applied to amino acids in whole blood at concentrations ranging from 0.18 (Leu + Ile) to 7.98 (Phe) of cut-off values, which should be sufficient for a biochemical test for inborn errors of amino acid metabolism [3,32].

Deng and Deng [5] reported that amino acids in blood were measured using the blood filter paper technique similar to our method. Amino acids were derivatized by n-buthanol and trifluoroacetic acid. The repeatability was lower than 5%, which was similar to our results, but the detection limits were lower than ours. The supposed reason is that the diameter of the punch (8 mm) was larger than ours (1/8 in.). However, the linearity of calibration curves ranged from 0.988 to 0.998, which were not good compared to ours. As those results, isotope dilution method is superior to non-isotope method for a quantitative calculation.

The method developed in this study was applied to five blood spot samples obtained from patients with inborn errors of amino acid metabolism, including PKU, MSUD and hypermethionine NICCD (Table 5). PKU is characterized by an increasing concentration of phenylalanine in the blood. Our results showed that the concentration of Phe was 1.96 and 6.01 times higher than the cut-off value. In maple syrup urine disease (MSUD), Leu, Ile, and Val accumulate in the blood. Our results showed the concentration of Leu was 13.07 and 5.02 times higher and that of Ile was 1.27 and 2.03 times higher than the cut-off values. In hypermethionine NICCD, phenylalanine, galactose, methionine or threonine increase in the blood. In this study, samples from a hypermethionine NICCD patient exhibited a concentration of Met that was 5.39 times higher than the cut-off value. These results show that this method can be applied to the chemical diagnosis of inborn errors of amino acid metabolism through the determination of the concentrations of the amino acids that are characteristically higher when these diseases are present.

The MS/MS method is superior to other methods in analysis time (only 2 min) and less expensive due to the application of flow injection as a method of sample introduction in MS/MS. For these reasons, the MS/MS method is widely applied to neonatal screening for inborn errors of amino acid, organic acid and fatty acid metabolism [1,5,6]. However, the GC/MS method has several aspects that are superior to the MS/MS method. In the MS/MS method, Leu and Ile are detected at the same *m/z* value without chromatographic separation and cannot be separated and determined individually. Ion-suppression effects due to co-eluting matrix components are not negligible in the MS/MS method, which prevents precise determination of analytes [33–35]. GC/MS can be used to avoid possible matrix effects that are detected by the MS/MS because the GC/MS can separate target compounds from the sample matrix with high chromatographic resolution. Electron ionization (EI)-GC/MS is also more resistant to ion-suppression than electrospray ionization-MS/MS. The characteristic mass spectral pattern obtained by EI can provide the mass numbers in the target compound, which do not overlap with other substances, so target compounds can be detected selectively. These advantages indicate that the GC/MS method is more appropriate for analyses in which lower analytical errors are required, such as for therapy monitoring and for specific patient diagnosis (e.g. moderate hyper-excretions or not an acute episode). The GC/MS method is necessary as a back-up method for MS/MS, especially as a precise quantitative method. In clinical laboratories, GC/MS is already widely used for various analyses, such as for organic acids in urine and for very long chain fatty acids in plasma that are indicative of an inborn error of metabolism [19,36–38]; thus, this method of amino acid analysis using GC/MS would be useful for those laboratories[9].

5. Conclusion

This new method enables simple, rapid and precise analysis for determination of amino acids in whole blood using GC/MS. It was successfully applied to 5 patients with 3 types of amino acid disorders, providing similar concentration levels to those reported using other methods.

Our study demonstrated the feasibility of routine biochemical test of amino acids using this method. Therefore, further studies to expand other amino acids should be meaningful in order to apply this method to routine biochemical tests for inborn errors of amino acid metabolism.

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References

[1] D.H. Chace, T.A. Kalas, E.W. Naylor, Clin. Chem. 49 (2003) 1797.
[2] A.A. Reilly, R. Bellisario, K.A. Pass, Clin. Chem. 44 (1998) 317.
[3] M.S. Rashed, M.P. Bucknall, D. Little, A. Awad, M. Jacob, M. Alamoudi, M. Alwattar, P.T. Ozand, Clin. Chem. 43 (1997) 1129.
[4] A. Jiye, J. Trygg, J. Gullberg, A.I. Johansson, P. Jonsson, H. Antti, S.L. Marklund, T. Moritz, Anal. Chem. 77 (2005) 8086.
[5] C. Deng, Y. Deng, J. Chromatogr. B 792 (2003) 261.
[6] H. Nishio, S. Kodama, S. Yokoyama, T. Matsuo, T. Mio, K. Sumino, Clin. Chim. Acta 159 (1986) 77.
[7] D. Oglesbee, K.A. Sanders, J.M. Lacey, M.J. Magera, B. Casetta, K.A. Strauss, S. Tortorelli, P. Rinaldo, D. Matern, Clin. Chem. 54 (2008) 542.
[8] P. Hušek, J. Chromatogr. B 669 (1995) 352.
[9] C. Deng, N. Li, X. Zhang, Rapid Commun. Mass Spectrom. 18 (2004) 2558.
[10] A.G. Calder, K.E. Garden, S.E. Anderson, G.E. Lobley, Rapid Commun. Mass Spectrom. 13 (1999) 2080.
[11] R.J.W. Truscott, D. Malegan, E. McCairns, B. Halpern, J. Hammond, R.G.H. Cotton, J.F.B. Mercer, S. Hunt, J.G. Rogers, D.M. Danks, Biomed. Mass Spectrom. 8 (1981) 99.
[12] H. Frank, A. Rettenmeier, H. Weicker, G.J. Nicholson, E. Bayer, Clin. Chim. Acta 105 (1980) 201.
[13] T.L. Perry, S. Hansen, Clin. Chim. Acta 25 (1969) 53.
[14] M.D. Armstrong, U. Stave, Metabolism 22 (1973) 549.
[15] J. Dallüge, R.J.J. Vreuls, D.J. van Iperen, M. van Rijn, U.A.Th. Brinkman, J. Sep. Sci. 25 (2002) 608.
[16] C. Deng, C. Shang, Y. Hu, X. Zhang, J. Chromatogr. B 775 (2002) 115.
[17] G.W. Lynes, M. Hjelm, J. Chromatogr. 562 (1991) 213.
[18] C. Deng, Y. Deng, B. Wang, X. Yang, J. Chromatogr. B 780 (2002) 407.
[19] I. Matsumoto, T. Kuhara, Mass Spectrom. Rev. 15 (1996) 43.
[20] T. Ohie, X.W. Fu, M. Iga, M. Kimura, S. Yamaguchi, J. Chromatogr. B 746 (2000) 63.
[21] I. MolnaÂr-Perl, Z.F. Katona, Chromatographia 51 (2000) S-228.
[22] T.G. Sobolevsky, A.I. Revelsky, I.A. Revelsky, B. Miller, V. Oriedo, J. Chromatogr. B 800 (2004) 101.
[23] P. Hušek, J. Chromatogr. 552 (1991) 289.
[24] J. Wang, Z. Huang, D.A. Gage, J.T. Watson, J. Chromatogr. A 663 (1994) 71.
[25] M. Vincenti, C. Minero, S. Lago, C. Rovida, J. High Resol. Chromatogr. 18 (1995) 359.
[26] D.R. Knapp, Handbook of Analytical Derivatization Reactions, John Wiley & Sons, Inc., New York, 1979.
[27] K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, 2nd ed., John Wiley & Sons Ltd., Chichester, UK, 1993.
[28] M.W. Haymond, C.P. Howard, J.M. Miles, J.E. Gerich, J. Chromatogr. 183 (1980) 403.
[29] S. Kawana, K. Katsuhiro, Y. Hasegawa, H. Kobayashi, S. Yamaguchi, Clin. Chim. Acta 392 (2008) 34.
[30] T.G. Sobolevsky, A.I. Revelsky, B. Miller, V. Oriedo, E.S. Chernetsova, I.G. Revelsky, J. Sep. Sci. 26 (2003) 1474.
[31] T. Kuhara, J. Chromatogr. B 758 (2001) 3.
[32] R. Blau, M. Duran, M.E. Blaskovics, K.M. Gibson, Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, 2nd ed., Springer, New York, 2003.
[33] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[34] H.R. Liang, R.L. Foltz, M. Meng, P. Bennett, Rapid Commun. Mass Spectrom. 17 (2003) 2815.
[35] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[36] S.I. Goodman, S.P. Markey, Diagnosis of Organic Acidemias by Gas Chromatograpy–Mass Spectrometry, Alan R. Liss, Inc., New York, 1981.
[37] P. Vreken, A.E.M. van Lint, A.H. Bootsma, H. Overmars, R.J.A. Wanders, A.H. van Gennip, J. Chromatogr. B 713 (1998) 281.
[38] M. Kimura, T. Yamamoto, S. Yamaguchi, Tohoku J. Exp. Med. 188 (1999) 317.

＜診断へのアプローチ —first line 検査＞

アシドーシス，ケトーシス

深尾敏幸*

はじめに

First line 検査としての血液ガス分析は血糖，血液ガス，アンモニアという 3 点セットのひとつであり，いわゆる急性発症型（けいれん，嘔吐，多呼吸，意識障害など脳炎や脳症を疑わせる症状）をとるタイプの代謝異常症のスクリーニングの必須項目である。またケトーシスの有無はアシドーシスの鑑別に重要であり，遊離脂肪酸とケトン体分画を一緒に測定する。

I 代謝性アシドーシス

一般には血液ガスは動脈採血であるが，よほどの循環不全がなければ，代謝疾患の評価には静脈血でもよい（通常の静脈採血での動脈血との pH，HCO_3^- の差が代謝異常症の診断に影響をあたえる可能性はきわめて少ない）。静脈血でもいいから検査しておくことが重要である。一般に，pH 7.30 未満，HCO_3^- 15 mmol/L 未満は代謝性アシドーシスが強いと考える。

代謝性アシドーシスは小児科においてよく認められる所見であり，先天代謝異常症がなくても，感染，異化亢進状態，脱水，組織低酸素状態などでも認められる。一方，代謝性アシドーシスをきたす先天代謝異常症は上述のような状態（感染，異化亢進など）で発症することも多い。代謝性アシドーシスの存在と評価は血液ガス分析を行うことが必須であり，病態の解釈には電解質，血糖，乳酸ピルビン酸，ケトン体，遊離脂肪酸，アンモニアとの同時測定することが望ましい。多くの場合は後に述べるケトーシスを合併している。

図 1 に，代謝性アシドーシス（pH <7.3，HCO_3^- <15 mmol/L）の鑑別フローチャートを示す。これは Saudubray らのアルゴリズムを改変したもの[1]であるが，血液ガス，血糖，アンモニア，乳酸の値に基づいている。最初にケトーシスの有無で大きく分けられている。原図ではケトーシスの「ある」・「なし」で分類されていたが，ここではあえて括弧でケトーシス「強い」・「軽い」とした。状況から予想されるよりケトーシスの程度が軽い場合は，これまでのケトーシス「あり」での検討でなく，「なし」の方向での検討が必要であるためである。低血糖をきたしている症例で，血中ケトン体が 800 μmol/L であれば，低血糖時なのにケトーシスは軽いとして「なし」の方向に進まないと脂肪酸 β 酸化系異常症にたどりつかない。

代謝性アシドーシスは呼吸性に代償されるため，多呼吸を生じる。このため代謝性アシドーシスが強ければ，患者は努力呼吸，多呼吸を呈する。図 2 はその代償のモデルである。もし呼吸性代償がおきないと，血液 pH はすぐに低下するが，呼吸性代償により pH が 7.3 を大きく切ることは少ない。代償しきれずに pH が 7.3 を切ってくるとき，生理的ではない病的な代謝状態を考えなくてはいけない。それが上述の鑑別フローチャートで，pH <7.3，HCO_3^- <15 mmol/L となっている理由である。

代謝性アシドーシスの評価では，アニオン

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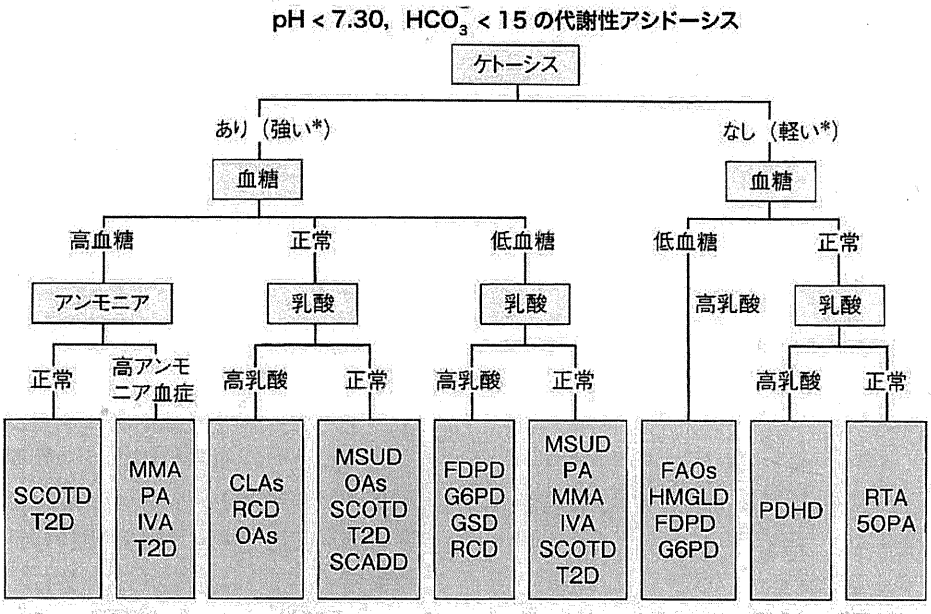

図 1　代謝性アシドーシス鑑別のフローチャート（Saudubray ら[1] 2001 の図 66-7 を改変）
SCOTD：サクシニル-CoA：3-ケト酸 CoA トランスフェラーゼ欠損症，T2D：ミトコンドリアアセトアセチル-CoA チオラーゼ欠損症，MMA：メチルマロン酸血症，PA：プロピオン酸血症，IVA：イソ吉草酸血症，CLAs：先天性高乳酸血症（ピルビン酸カルボキシラーゼ欠損症，マルチプルカルボキシラーゼ欠損症，ケトグルタル酸脱水素酵素欠損症，E3 欠損症など），RCD：呼吸鎖異常症，OAs：有機酸代謝異常症，MSUD：メープルシロップ尿症，SCADD：短鎖アシル-CoA 脱水素酵素欠損症，FDPD：フルクトースビスホスファターゼ欠損症，G6PD：グルコース 6 ホスファターゼ欠損症，GSD：グリコーゲン合成酵素欠損症，PDHD：ピルビン酸脱水素酵素欠損症，RTA：腎尿細管性アシドーシス，5OPA：5-オキソプロリン尿症　* 本文参照

A.

$$pH = 6.1 + \log\frac{HCO_3}{S \cdot PCO_2}$$

$$= 7.62 + \log\frac{HCO_3}{PCO_2}$$

B.

HCO_3 (mM)	24	15	**15**	10	**10**	5	**5**
PCO_2 (mmHg)	40	40	**28**	40	**22**	40	**16**
pH	7.40	7.19	**7.35**	7.02	**7.28**	6.72	**7.11**

図 2　代謝性アシドーシスと呼吸性代償
A：ヘンダーソン・ハッセルバルヒの式　B：血中の HCO_3^- と PCO_2 と pH の関係
太字は呼吸性代償が生じている場合を示す。

ギャップをみることも鑑別には有用である。通常，血液ガスを測定する状況では電解質も測定されているはずである。アニオンギャップ＝Na－（HCO_3＋Cl）で，正常値は 10～14 mEq である。これが増大していることは，HCO_3^-，Cl 以外の陰性イオン（酸）が蓄積していることを意味する。

2 次，3 次スクリーニングとして，尿有機酸スクリーニングやタンデムマスによる代謝スクリーニングを行う。発作時の検体でないと診断ができない疾患や例があり，発作時の尿，血清の凍結，濾紙血採取が重要である。

II ケトーシス

一般に，アセト酢酸，3-ヒドロキシ酪酸，アセトンを総称してケトン体という。アセトンはアセト酢酸が脱炭酸して生じ，代謝的には大きな意味をもたず，呼気に排泄され，アセトン臭をきたす原因である。血液ケトン体分画は，アセト酢酸と 3-ヒドロキシ酪酸の 2 つを指し，ともにかなり強い酸であり，血液中に蓄積すればアシドーシスをきたす。

ケトン体はグルコースをセーブするための代替エネルギー源である。脂肪組織から遊離脂肪酸が切り出され，それが肝臓のミトコンドリアに取り込まれ，脂肪酸β酸化，HMG-CoAを経て，ケトン体が産生される。そのため遊離脂肪酸の動員とケトン体の動員は本来一連のことである。ケトン体産生において，①脂肪組織における遊離脂肪酸の放出，②肝臓ミトコンドリアへの脂肪酸の取り込み，③HMG-CoA合成の3ステップにおいてホルモン調節を受けており，インスリンは3ステップを強く抑え，カテコールアミン，グルカゴンは促進する。このためインスリンが優位な食後や高インスリン血症では，脂肪酸動員，ケトン体産生は抑制され，カテコールアミン，グルカゴンが優位な空腹，感染，ストレスなどの状況では脂肪酸動員，ケトン体産生は亢進する。

小児ではケトーシスを生じやすい。それは脂肪酸β酸化系-ケトン体産生系が小児における血糖維持に重要であることによる。そのため急性胃腸炎や気管支炎などで食欲が落ち，発熱などがあればすぐにケトーシスとなる。また小児では，いわゆるアセトン血性嘔吐症，ケトン血性低血糖症など名前にケトンのついた病態も存在する。これらの疾患はケトーシスをきたす頻度として多く，またケトーシス時に嘔吐，嗜眠傾向があり，発作を反復することから，臨床的に重篤感がある場合は代謝異常症の可能性も考えて十分な代謝的検討が行われるべきである。

ケトーシスの評価には尿ケトンがスクリーニングとして用いられているが，血中の遊離脂肪酸とケトン体分画を測定することが重要である。どの程度のストレス（空腹，発熱）がどの程度持続して存在した状況でのケトン体の値なのか？ 血糖と遊離脂肪酸がどの程度のときの値なのかを常に考える必要がある。24時間飢餓テスト時の遊離脂肪酸やケトン体の変化についての報告は参考になる[2]（表）。もちろん発熱，感染などが加われば，さらにケトン体は高くなるはずである。

ケトーシスの臨床的重要性は大きく2つに分類できる。ひとつは本来飢餓，発熱などケトーシスがあるべき状況でケトーシスがない場合である。低血糖があって，本来であればケトーシスが強いはずなのにケトーシスが軽いという低ケトン性低血糖では，脂肪酸酸化異常症，ケトン体産生障害，もしくはインスリン過分泌状態などが考えられる。ふたつめはケトーシスが強く，ケトアシドーシス（血中ケトン体7000 μmol/L以上）の場合である。この場合は異化の著しい亢進状態，有機酸代謝異常症，ケトン体利用障害などを考える必要がある[3,4]。

まれではあるが，ケトン体産生系は正常で末梢組織がケトン体を利用できないケトン体利用障害

表　空腹負荷テスト時の各種検査値の動き

	生後1か月～1歳（n=12）			1歳～7歳（n=27）			7歳～15歳（n=9）		
	15時間	20時間	24時間	15時間	20時間	24時間	15時間	20時間	24時間
血糖	70.2～95.4	63.0～82.8	48.6～81.0	63.0～86.4	50.4～77.4	50.4～68.4	79.2～88.2	68.4～88.2	54～77.4
(mg/dl)	84.6	70.2	64.8	79.2	63.0	59.4	84.6	77.4	68.4
遊離脂肪酸	0.5～1.6	0.6～1.3	1.1～1.6	0.6～1.5	0.9～2.6	1.1～2.8	0.2～1.1	0.6～1.3	1.0～1.8
(mmol/L)	1.0	0.9	1.3	1.1	1.7	2.1	0.7	1.0	1.4
総ケトン体	0.1～1.5	0.6～3.2	1.5～3.9	0.15～2.0	1.2～3.7	2.2～5.8	<0.1～0.5	0.1～1.3	0.7～3.7
(mmol/L)	0.4	1.6	2.7	0.8	2.4	3.5	0.2	0.6	1.3
3-ヒドロキシ酪酸	0.1～1.0	0.5～2.3	1.1～2.8	<0.1～0.9	0.8～2.6	1.7～3.2	<0.1～0.3	<0.1～0.8	0.5～1.3
(mmol/L)	0.4	1.1	1.8	0.6	1.8	2.5	0.1	0.4	0.9
遊離脂肪酸/総ケトン体	0.6～5.2	0.3～1.4	0.3～0.7	0.7～4.0	0.4～1.5	0.4～0.9	1.9～10.0	0.7～4.6	0.5～2.0
	2.3	0.8	0.9	2.2	0.8	0.6	5.4	2.5	1.5
乳酸	9.9～20.7	7.7～16.2	7.2～18.0	7.2～13.5	4.5～15.3	6.3～14.4	5.4～8.1	5.4～8.1	3.6～8.1
(mg/dl)	16.2	11.7	12.6	9.0	9.9	10.8	8.1	6.3	6.3

上段は10～90パーセンタイルを示し，下段は中央値を示す。
ケトン体は単位がmmol/Lである。7歳以降では1～7歳に比べケトン体の値が低くなっている。
空腹試験は専門医のもとで慎重に行うべきものである。
（Bonnefontら[2] 1990より一部改変）

の疾患（ミトコンドリアアセトアセチル-CoA チオラーゼ〔T2〕欠損症，サクシニル-CoA：3-ケト酸 CoA トランスフェラーゼ〔SCOT〕欠損症）がある。アセトン血性嘔吐症やケトン血性低血糖症の発作時はケトン体産生亢進によるケトーシスであり，T2 欠損症や SCOT 欠損症などのケトン体分解異常症はその産生亢進プラス利用障害によるケトアシドーシスであり，誘因などは共通である。そのため，最も重要な鑑別点はアシドーシスの程度といえる。また，アセトン血性嘔吐症やケトン血性低血糖症は 1 歳半以降の発症がほとんどであるが，ケトン体分解異常症の発症はそれ以前にピークがある。そのため，重いアセトン血性嘔吐症様の 1 歳の児をみたら基礎疾患を疑うべきである。

1）尿ケトン体

First line の臨床検査として，尿ケトン定性がある。この方法はアセト酢酸に反応するが，3-ヒドロキシ酪酸には反応しないことに注意。一般には，尿ケトンは血中ケトン体増加を反映するが，代謝評価には血中ケトン体分画の測定が重要である。尿ケトン陰性例でも血中の 3 ヒドロキシ酪酸が 1000 μmol/L をこえる場合もある。尿を凍結保存しておくことが，その後の診断に役立つ。ほとんど飲めず食べられずの胃腸炎の児は尿ケトン体が陽性であるのが普通である。低血糖でけいれんを起こした児が尿ケトン陰性であれば，疾患はしぼられる。

2）血中ケトン体分画，遊離脂肪酸

血清ではケトン体分画としてアセト酢酸と 3-ヒドロキシ酪酸の両者が測定できる。評価には遊離脂肪酸を同時測定することが必要である。

一般に記されている正常値は早朝空腹時採血で，総ケトン体 130 μmol/L 以下（アセト酢酸 55 μmol/L 以下，3-ヒドロキシ酪酸 85 μmol/L 以下）となっているが，小児では年齢や空腹時間で大きく変化する。ケトン体は食後などは 100 μmol/L 以下となるが，24 時間空腹で幼児期では 6000 μmol/L 近くまで増加する。健常者でも実に 100 倍近く増加することになる。一般に，総ケトン体が 7 mM（7000 μmol/L）以上をケトアシドーシスといい，0.2 mM（200 μmol/L）以上をケトーシスという。遊離脂肪酸/総ケトン体比もしくは遊離脂肪酸/3-ヒドロキシ酪酸比をみる（単位をともに mmol/L にそろえて比較）ことが評価に必要であり，脂肪酸酸化異常症，ケトン体産生異常症では，遊離脂肪酸は動員されてもケトン体が産生されず，遊離脂肪酸/総ケトン体比は 20 をこえる。一方，ケトン体利用障害では空腹のかなり初期から遊離脂肪酸/総ケトン体比は 0.3 以下になる。

Key Points

❶静脈血でもいいので血液ガス検査をすることが重要。一般に pH 7.30 未満，HCO_3^- 15 mmol/L 未満は代謝性アシドーシスが強いと考える。

❷代謝性アシドーシスの鑑別には，血糖，アンモニア，血中ケトン体（プラス遊離脂肪酸），乳酸が役立つ。

❸2 次，3 次スクリーニングとして尿有機酸スクリーニング，タンデムマスによる代謝スクリーニングを行うための検体保存が重要である。

❹ケトーシスの重要性は，①本来飢餓，発熱などケトーシスがあるべき状況でケトーシスがない場合と，②ケトーシスが強く，ケトアシドーシス（血中ケトン体 7000 μmol/L 以上）の場合がある。

❺ケトン体の評価には遊離脂肪酸の同時測定が必要である。

文　献

1) Saudubray J-M, Charpentier C : Clinical phenotypes : diagnosis/algorithms. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds) : Metabolic and Molecular Bases of Inherited Disease, 8th ed, McGraw-Hill, New York pp1327-1403, 2001
2) Bonnefont JP, et al : The fasting test in paediatrics : application to the diagnosis of pathological hypo- and hyperketotic states. Eur J Pediatr **150** : 80-85, 1990
3) Mitchell GA, Fukao T : Inborn errors of ketone body metabolism. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds) : Metabolic and Molecular Bases of Inherited Disease, 8th ed, McGraw-Hill, New York, p2327-2356, 2001
4) 深尾敏幸：ケトン体代謝異常症：特にアセトン血性嘔吐症と鑑別すべきサクシニル-CoA：3-ケト酸 CoA トランスフェラーゼ欠損症を中心に．日小児会誌 **111** : 723-739, 2007

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Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency

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ABSTRACT

Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency is an inborn error of ketone body metabolism and causes episodic ketoacidosis. We report clinical and molecular analyses of 5 patients with SCOT deficiency. Patients GS07, GS13, and GS14 are homozygotes of S405P, L327P, and R468C, respectively. GS17 and GS18 are compound heterozygotes for S226N and A215V, and V404F and E273X, respectively. These mutations have not been reported previously. Missense mutations were further characterized by transient expression analysis of mutant cDNAs. Among 6 missense mutations, mutants L327P, R468C, and A215V retained some residual activities and their mutant proteins were detected in immunoblot analysis following expression at 37 °C. They were more stable at 30 °C than 37 °C, indicating their temperature sensitive character. The R468C mutant is a distinct temperature sensitive mutant which retained 12% and 51% of wild-type residual activities at 37 and 30 °C, respectively. The S226N mutant protein was detected but retained no residual activity. Effects of missense mutations were predicted from the tertiary structure of the SCOT molecule. Main effects of these mutations were destabilization of SCOT molecules, and some of them also affected catalytic activity. Among 5 patients, GS07 and GS18 had null mutations in both alleles and the other three patients retained some residual SCOT activities. All 5 developed a first severe ketoacidotic crisis with blood gas pH <7.1, and experienced multiple ketoacidotic decompensations (two of them had seven such episodes). In general, the outcome was good even following multiple ketoacidotic events. Permanent ketosis or ketonuria is considered a pathognomonic feature of SCOT deficiency. However, this condition depends not only on residual activity but also on environmental factors.

1. Introduction

Ketone bodies, produced mainly in the liver, are an important source of energy for extrahepatic tissues [1]. Succinyl-CoA: 3-ketoacid CoA transferase (SCOT; EC 2.8.3.5, gene symbol OXCT1) is a mitochondrial homodimer essential for ketone body utilization. SCOT catalyzes acetoacetate activation to acetoacetyl-CoA in mitochondria. The human OXCT1 gene is mapped to 5p13 and consists of 17 exons [2,3]. Human SCOT cDNA encodes a precursor subunit of 520 amino acids.

Patients with SCOT deficiency (OMIM 245050) experience episodic ketoacidosis and are usually asymptomatic between episodes. Fewer than 30 affected individuals are known [2–22]. Urinary organic acid analysis and acylcarnitine analysis show non-specific profiles in this disorder. Hence, in vitro methods of diagnosis, such as enzyme assay and mutation analysis, are essential for the definite diagnosis. Permanent ketosis or ketonuria is a pathognomonic feature of this disorder but is not always present [17,20,22]. We previously identified 11 mutations of the OXCT1 gene [6,8–14] in 12 SCOT-deficient families.

Recently, the human SCOT tertiary structure has become available (PDB entry 3DLX). This has enabled us to evaluate effects of missense

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mutations more precisely than homology modeling using porcine SCOT structure [20].

We herein describe 5 SCOT-deficient patients and characterize their mutations by transient expression analysis of mutant cDNAs and discuss the mutation sites on the tertiary structure of human SCOT.

2. Patients, materials and methods

2.1. Patients

GS07: Clinical findings of GS07 have been reported previously [11]. Briefly, he presented with two episodes of ketoacidosis during infections at 17 and 25 months of age. He was followed until age 6 years without any further episodes.

GS13: Some aspects of GS13 have already been published [14]. Although parents are not known to be related, the family originates from a small socially isolated area, making consanguinity not unlikely. After an initial severe crisis at age 6 months, the girl had three additional, but milder crises during infancy. After 6 h of fasting during a test performed after the first crisis, her blood pH was 7.42, HCO_3 12.5 mmol/L and BE −9.1 mmol/L, with high serum ketones 3.55 mmol/L and low FFA of 0.29 mmol/L. At the age of 12.5 years she is now doing well, attending a regular school. She usually takes her last meal at about 10 p.m. and takes first morning meal in school at about 9 to 10 a.m. without any signs or symptoms. Acid-base balance was checked three times at about 8 a.m. and was always normal.

GS14: This female patient of 21 years of age was born to consanguineous parents in October 1988. She had many hospitalizations in the pediatric ward for episodes of hypoglycemia. Her first hospitalization was at 19 months for seizures and coma. On physical exam she had deep respiration. Her blood glucose was 1.3 mmol/L, She had severe metabolic acidosis with pH 6.93, HCO_3 6 mmol/L and ketonuria 3+. She had six similar episodes of hypoglycemia with ketoacidosis without seizures, usually after episodes of infections. Her neuro-developmental status was normal. She had persistent ketonuria between episodes of decompensation.

GS17: She is a Caucasian girl born to non-consanguineous parents at full-term with a birth weight of 2230 g. She had low blood glucose levels of 1.6 mmol/L on the first day of life and received intravenous glucose for 1–2 days. She was discharged on day 5 of life. She was well until 3 years of age when she developed tachypnea and lethargy following gastroenteritis. Her blood pH was 6.99 with BE −25 mmol/L and her blood glucose was 7.5 mmol/L. Urine analysis showed massive ketones. She was intubated and admitted to the intensive care unit. She responded to intravenous glucose and bicarbonate and was discharged after 7 days. Six months later she was readmitted to a local hospital with mild lethargy, tachypnea and ketoacidosis which developed during a febrile upper respiratory infection. The patient's mother reported that the patient has had trace to moderate ketonuria, even when she was in good health. SCOT deficiency was confirmed by an enzyme assay using fibroblasts. The patient has not had any more episodes of ketoacidosis for 6 years. She is doing well and receives a mildly protein-restricted diet (2.0 g/kg/day), avoids prolonged fasting and adheres to "sick day" precautions such as increasing calories from carbohydrates in her diet and intravenous glucose as needed.

GS18: The male patient, first child of healthy non-consanguineous parents from Vietnam, was born at 39 weeks with a birth weight of 3390 g. After a normal clinical presentation during the first days of life he was readmitted to hospital at the age of 3 days with polypnea. Biochemically he presented with severe metabolic acidosis (pH 7.08, pCO_2 25 mmHg, BE −22.6 mmol/l) and pronounced ketonuria. Beside ketones, metabolic screening revealed unremarkable urinary organic acids. With intravenous fluid with sodium bicarbonate the patient recovered within hours. During the first year of life the patient was hospitalized three times because of episodes of severe ketoacidosis (minimal pH 6.98, pCO_2 15 mmHg, BE −28 mmol/l). At the age of 1 year, SCOT deficiency was confirmed by an enzyme assay using his lymphocytes and platelets. He was on treatment that consisted of avoidance of prolonged fasting and moderate protein restriction (1.5 g/kg/d). Subsequently the patient has had three more severe episodes of ketoacidosis in the course of intercurrent diseases. Otherwise, he has permanent mild ketonuria. At his present age of 10 years psychomotor and physical development are normal.

Table 1 summarizes the clinical presentations and laboratory data of these 5 SCOT-deficient patients. This study has been approved by The Ethical Committee of Graduate School of Medicine, Gifu University.

2.2. Enzyme assay and immunoblot analysis

Assays for acetoacetyl-CoA thiolase and for SCOT were performed as described [7,23], using acetoacetyl-CoA as a substrate and measuring its disappearance spectrophotometrically.

2.3. Mutation analysis

Total RNA was purified from peripheral blood mononuclear cells with an ISOGEN kit (Nippon Gene, Tokyo, Japan). RT-PCR was as described [2]. Mutations were detected by amplifying cDNA spanning the full-length coding sequence, and sequencing more than 5 clones.

Genomic DNA was purified with a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). Mutation analysis at the genomic level was done by PCR for each exon and its intron boundaries (at least 20 bases from the exon/intron boundaries for both directions) followed by direct sequencing [3].

2.4. Construction of eukaryote transient expression vectors

Wild-type full-length SCOT cDNAs [3] were subcloned into the pTZ18U and pCAGGS eukaryote expression vectors [24] and designated the pTscotWild-type and pCAGGSscotWild-type, respectively. Mutations were introduced into the pTscotWild-type using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla CA), confirmed by sequencing, and then transferred into pCAGGS.

2.5. Transient expression analysis

Wild-type and mutant SCOT expression vectors (4 μg) were first transfected using Lipofectamine 2000 (GIBCO BRL Invitrogen Inc., Carlsbad, CA) in ~10^5 SV40-transformed SCOT-deficient fibroblasts of GS01[2]. One microgram of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct [25], was cotransfected to monitor transfection efficiency. Transfection was done at 37 °C for 24 h was followed by a further 48-h incubation at 37 or 30 °C. The cells were harvested and stored at −80 °C until SCOT and CT activities were assayed. Immunoblot was done using a mixture of anti-[human SCOT] antibody and anti-[human CT] antibody as the first antibody [26]. The quantity of mutant protein was estimated densitometrically, and was compared to the signal intensities of serially diluted samples of the wild-type SCOT protein.

2.6. Tertiary structural model of human SCOT

To analyze the putative structural implications of the SCOT mutations, the recently determined crystal structure from the Structural Genomics Consortium (PDB entry 3DLX) of human SCOT was taken as a starting point. Prior to the analysis, the structure was subjected to further refinement in PHENIX [27] and COOT [28], including the addition of missing side chains and rebuilding of the solvent network. The figures describing the structural details were prepared with PyMOL.

Table 1
Clinical presentation and mutation of SCOT-deficient cases.

GS number	Nationality	Sex	Consanguinity	Affected siblings	First ketoacidotic crisis						Persistent ketonuria	Frequency of crisis	Present age	Prognosis	Gene mutation		SNP		
					Onset	Symptom	Blood gas pH	HCO3	BE	Glucose					Paternal	Maternal	rs75134564 c.173C>T(T58M)	rs1136453 c.315 G>C(S105S)	c.1497 A>G(Q499Q)
GS07	France	M	+	–	1y5m	Polypnea, lethargy	6.92	4		na	+	2	(6y)	NP	c.1213T>C (S405P)	c.1213T>C (S405P)	C/C	C/C	G/G
GS13	Croatia	F	*	–	6 m	Appetite loss, coma	6.90	3.2	−27	7.8	–	2	12y	NP	c.980T>C (L327P)	c.980T>C (L327P)	C/C	C/C	G/G
GS14	Tunisia	F	+	–	1y7m	Seizure, coma	6.93	6		1.3	+	7	21y	NP	c.1402C>T (R468C)	c.1402C>T (R468C)	C/C	C/C	G/G
GS17	USA	F	–	–	3y	Polypnea	6.99	5	−25	7.5	+	2	9y	NP	c. 644C>T (A215V)	c.677G>A (S226N)	C/C	C/C	G/G
GS18	Germany**	F	–	+	3d	Polypnea, lethargy	7.08		−22.6	9.1	+	5	10y	NP	c.817G>T (E273X)	c.1210G>T (V404F)	C/T	G/G	A/A

NP: nothing particular.
* Possible consanguinity.
** GS18 is Vietnamese.

3. Results and discussion

3.1. Enzyme assay

Enzyme assay data for 4 patients are shown in Table 2. All four patients' fibroblasts presented with decreased SCOT activity, whereas they had a potassium-ion activated acetoacetyl-CoA thiolase activity which was a specific character of mitochondrial acetoacetyl-CoA thiolase (T2). In immunoblot analysis, SCOT protein was scarcely detected in these patients' cells, whereas T2 protein was clearly detected (data not shown). Lymphocytes and platelets from GS18 had no apparent SCOT activity (data not shown). These results confirmed the diagnosis of SCOT deficiency in the 5 patients.

3.2. Mutation analysis

Both genomic mutation analysis and cDNA analysis were done in all the cases except for GS18 of whom RNA was not available. The results of mutation analyses are shown in Table 1. Three patients with definite or possible consanguinity had homozygous mutations (c.1213T>C (S406P) in GS07; c.980T>C (L327P) in GS13; c.1402C>T (R468C) in GS14). GS17 is a compound heterozygote of c.644C>T (A215V) from the father and c.677G>A (S266N) from the mother. GS18 is also a compound heterozygote of c.817G>T (E273X) from the father and c.1210G>T (V404F) from the mother. We also detected three single nucleotide polymorphisms. Among them, c.173C>T (T58M) (rs75134564) was previously identified in a Japanese patient (GS02) and demonstrated not to reduce enzyme activity [13].

3.3. Transient expression analysis of mutant cDNAs

We performed transient expression analysis of wild-type and mutant cDNAs in SCOT-deficient SV40-transformed fibroblasts. Following expression of SCOT cDNAs for 48 h at either 37 or 30 °C, an enzyme assay and immunoblots were performed (Fig. 1). The transfection of wild-type SCOT cDNA produced high SCOT activity, whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. Among 6 missense mutations, S226N, V404F, and S405P did not retain residual SCOT activity. The A215V, L327P and R468C mutants retained detectable residual activities, 3.5, 4.7 and 12% of the wild-type value, respectively, in expression at 37 °C. Their relative residual SCOT activities to wild-type in expression at 30 °C were 3- to 4-fold higher than those in expression at 37 °C. In particular, R468C mutants retained a 51% activity of wild-type value in the expression at 30 °C. In immunoblot analysis, V404F and S405P protein was not detected in expression at 30 and 37 °C. S226N protein was clearly detected in the expression at 30 °C without any detectable residual activity, indicating that S226N protein was an inactive protein. The relative amount of the A215V, L327P, and R468C mutant proteins, as compared to the wild-type, was estimated to be 30%, 30%, and 50%, respectively, in expression at 30 °C. These proteins were more stable at 30 °C than at 37 °C. Specific activities (activity/protein) of A215V, L327P, and R468C mutants could be calculated to about 50%, 50%, and 100% that of wild-type, respectively.

Table 2
Enzyme assay using fibroblasts.

	Acetoacetyl-CoA thiolase			SCOT	SCOT/+K^+
	−K^+	+K^+	+K^+/−K^+		
GS07	5.9	12.9	2.2	1.9	0.2
GS13	4.3	9.1	2.1	0.8	0.1
GS14	3.6	7.3	2.0	1.2	0.2
GS17	7.0	14.9	2.1	1.2	0.1
Controls ($n=5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1	0.6 ± 0.2

Enzyme activity is expressed as nmol/min/mg protein.
Fibroblasts from GS18 were not available.

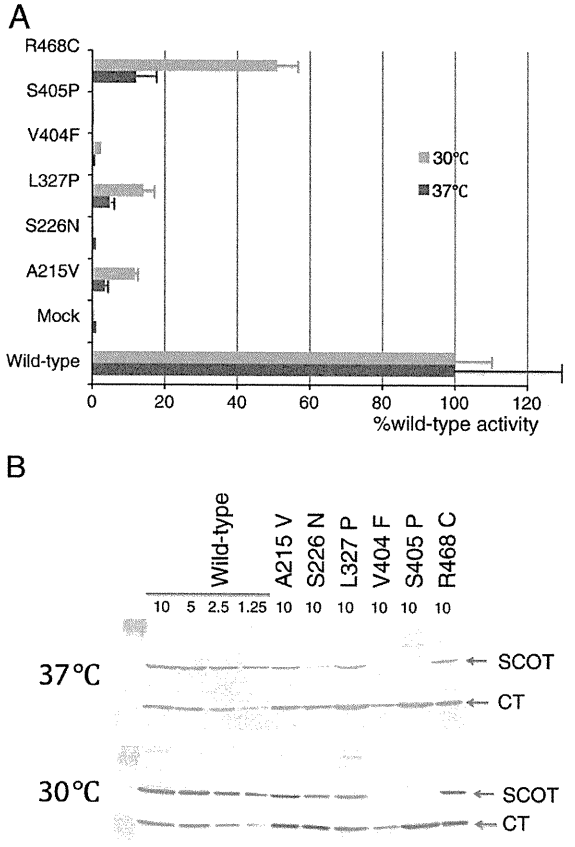

Fig. 1. Transient expression results for wild-type and mutant SCOT cDNAs. Wild-type and mutant SCOT expression vectors (4 μg) were transfected together with 1 μg of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct, to SV40-transformed fibroblasts of GS01 of which the mutation is S283X/S283X. Transient expression was done 37 and 30 °C. Mock, transfection of 1 μg of pCAGGSct and 4 μg of pCAGGS vectors without insert. (A) SCOT activities relative to those in wild-type transfection are shown. The mean values are displayed together with the SD of three independent experiments. (B) Immunoblots for SCOT and CT are shown. The protein amounts applied are shown above the lanes. The first antibody was a mixture of an anti-CT (cytosolic thiolase) antibody and anti-SCOT antibody. The positions of the bands for CT and SCOT are indicated by arrows.

3.4. Tertiary structural model of human SCOT and mutations

A number of mutations have been characterized for SCOT deficiency, and several of them have been structurally analyzed before [3,20,22], based on homology models of human SCOT, made with the help of the pig SCOT crystal structure [20]. Fig. 2A shows a dimer of human SCOT, with presently and previously identified mutations highlighted on the SCOT monomer in Fig. 2B. It is noteworthy that most of the mutations are located around two 'hot-spots' in 3D space; these areas correspond to a small beta sandwich domain in the N-terminal lobe, and a larger beta sandwich structure close to the C terminus. Sporadic mutations are also seen closer to the active site cavity.

The mutation A215V involves the residue A215, which in the wild-type protein is in the middle of a beta sheet, pointing inwards into the protein. The terminal carbon atom of A215 is only 3.6 Å away from the terminal methyl group carbons of L269 in an opposing beta sheet. Thus, even a small valine residue cannot be incorporated into this position without structural strain and changes. The position is located at a small beta sandwich domain involved in SCOT dimer formation. A215 is, furthermore, in the very close vicinity of the previously characterized SCOT mutations G219E and V221M [3]. These observations on the tertiary structure are in accord with the results that the main mutant effect of A215V is the instability of the mutant protein since the mutant protein amount was 12.5% and 30% relative to wild-type at 37 and 30 °C, respectively.

S226 is located close to the dimerization interface, although not being directly involved in it. The side chain is hydrogen-bonded to the backbone carbonyl of D362 and *via* a buried water molecule to N345; N345 is vicinal to the crucial catalytic residue E344 (Fig. 2D). Thus, the S226N mutation is likely to disturb the structure at least locally, and could also affect the properties of the catalytic site. As expected from the view of tertiary structure, the S226N mutant protein was revealed to be unstable and non-functional protein was detected in transient expression analysis.

L327 locates to an alpha helix on the SCOT surface, close to the active site entrance (Fig. 2D). The side chain of L327 is solvent-exposed and disordered in the crystal. This helix could form part of the CoA substrate-binding site, and a proline mutation in the central part of this helix may both perturb the helical structure and affect the functional mobility of this region, especially since the neighboring residue (326) is also a proline. A main mutant effect of L327P is the instability of the mutant protein since the mutant protein amount was 12.5% and 30% relative to wild-type at 37 and 30 °C, respectively.

V404 locates to the C-terminal beta sandwich domain of SCOT (Fig. 2E). The side chain points inwards, into a tightly packed hydrophobic core between two beta sheets. There clearly is no room for a large Phe residue, as there are several short distances from the side chain of V404 to residues from the opposing beta sheet. S405 is neighboring V404, at the end of a beta strand, preceding a short tight beta turn (Fig. 2E). In light of this, the side chain hydroxyl group is within hydrogen bonding distance from three main-chain NH groups from residues 407–409. Such an arrangement would be completely destroyed upon mutation of residue 405 to proline. As expected from the tertiary structure, these mutant proteins were too unstable to detect in either the 37 or 30 °C expression.

R468 is an exposed residue, present in the beta sheet opposite to that harboring V404 and S405. Its side chain is a central residue in a salt bridge network with E488, R308, E312, K436, and D477 (Fig. 2F). Mutation of the R468 residue will be detrimental to this large hydrophilic region on the surface of the C-terminal domain of SCOT. Since its specific activity is similar to wild-type, R468C does not affect catalytic activity; the active site residues are far from this mutation. The main mutational effect of R468C is also instability of the molecule.

3.5. Clinical phenotypes and genotypes

We reported herein 5 SCOT-deficient patients and their clinical and molecular aspects are summarized in Table 1. They developed the first ketoacidotic episodes from 3 days of age to 3 years of age. The episodes were associated with very severe metabolic acidosis with blood pH ranging from 6.90 to 7.08. They all recovered from the first ketoacidotic crises and were well managed after the diagnosis of SCOT deficiency was made. We previously reported clinical and molecular characters for 12 SCOT-deficient families and now have added to those 5 more.

Permanent ketosis or persistent ketonuria is pathognomonic feature of SCOT deficiency. We, however, previously showed that SCOT-deficient patients with "mild" mutations may have no permanent ketosis. V221M, R268H, or T435N homozygotes and T435N/null mutation compound heterozygotes did not show permanent ketosis or permanent ketonuria [3,17,20,22]. V221M, R268H and T435N mutations retained 10%, 34% and 25%, respectively, relative activity to wild-type in 37 °C expression using the same expression system. In the present study, L327P and R468H mutations retained 4.7% and 12%, respectively, relative activity in 37 °C expression. A L327P homozygote, GS13, did not have permanent ketonuria but a R468H homozygote; GS14 did. To our knowledge, patients with null mutations all showed permanent ketonemia or ketonuria. Hence "mild" mutation with

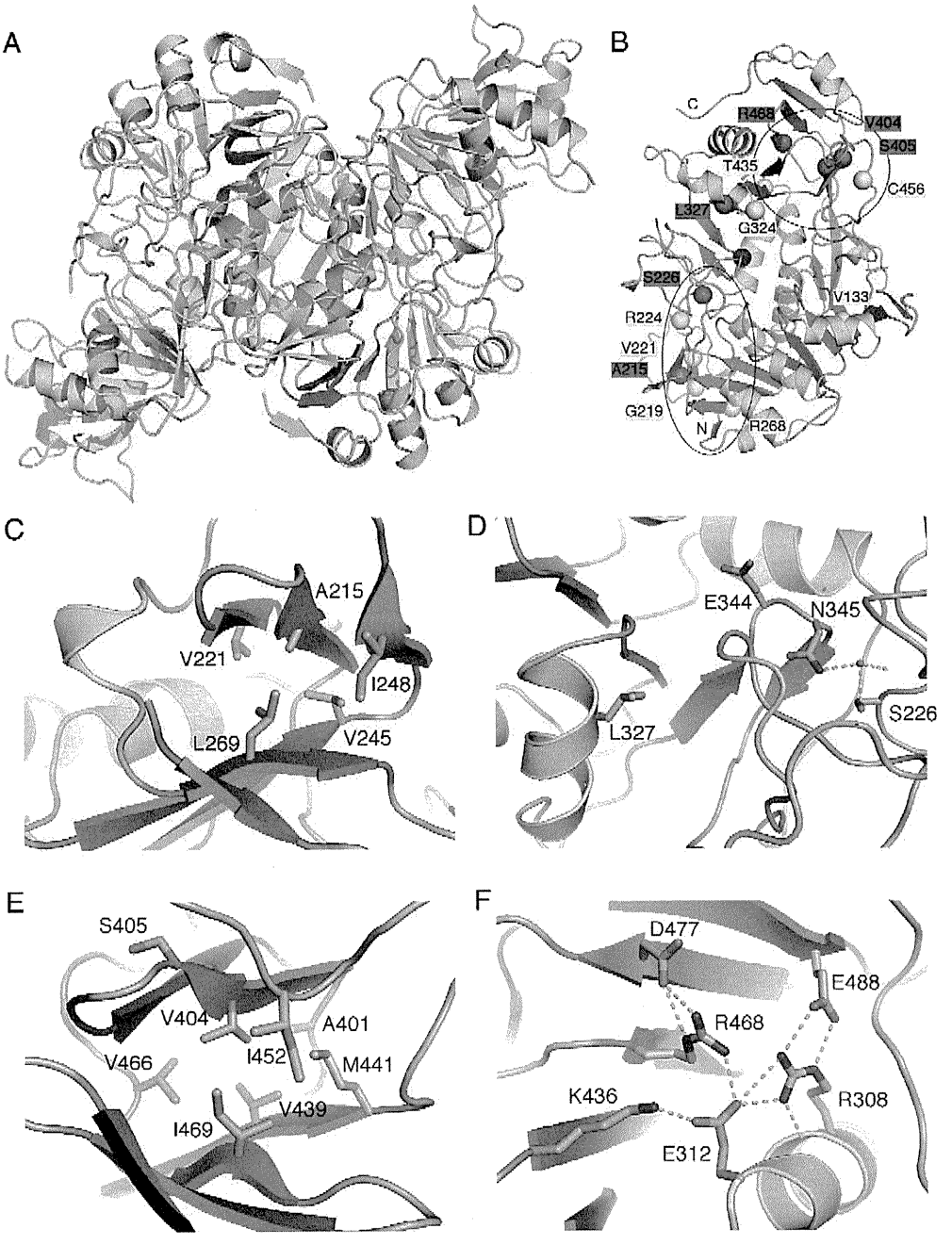

Fig. 2. Mutation sites on the tertiary structure of SCOT monomer. (A) An overall structure of human SCOT dimmer (PDB entry 3DLX). (B) A monomer of human SCOT. The N and C termini are labelled. The two clusters of mutations are indicated by ellipsoids. The positions of the mutations identified in this study are in red, and the ones previously identified are shown in yellow. The position of the catalytically active glutamate residue 344 is marked with a blue sphere. (C) The environment of the A215V mutation in the tightly packed hydrophobic core of the small beta sandwich. The N terminus of the crystal structure is at the bottom front. (D) S226 lies close to the active site, and interacts with N345 via a water-mediated hydrogen bond (green) and by van der Waals interactions. The catalytic residue is E344. L327 also lies close to the entrance of the catalytic cavity and could be involved in substrate binding. (E) V404 and S405 are next to each other, V404 being buried at the hydrophobic core of a beta sandwich unit. S405 interacts with the backbone amides of a tight turn (blue). (F) R468 plays a central role in a salt bridge network linking a beta sheet and a helix.

residual activity may be a necessary condition but not a sufficient condition for the absence of permanent ketonemia or ketonuria. Environmental factors may also affect a clinical phenotype of persistent ketonuria.

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References

[1] G.A. Mitchell, T. Fukao, Chapter 102. Inborn errors of ketone body catabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), Metabolic and molecular bases of inherited disease, 8th edition, McGraw-Hill, Inc, NewYork, 2001, pp. 2327–2356.

[2] S. Kassovska-Bratinova, T. Fukao, X.Q. Song, A.M. Duncan, H.S. Chen, M.F. Robert, C. Perez-Cerda, M. Ugarte, C. Chartrand, S. Vobecky, N. Kondo, G.A. Mitchell, Succinyl CoA: 3-oxoacid CoA transferase (SCOT): human cDNA cloning, human chromosomal mapping to 5p13, and mutation detection in a SCOT-deficient patient, Am. J. Hum. Genet. 59 (1996) 519–528.

[3] T. Fukao, G.A. Mitchell, X.Q. Song, H. Nakamura, S. Kassovska-Bratinova, K.E. Orii, J. E. Wraith, G. Besley, R.J. Wanders, K.E. Niezen-Koning, G.T. Berry, M. Palmieri, N. Kondo, Succinyl-CoA:3-ketoacid CoA transferase (SCOT): cloning of the human SCOT gene, tertiary structural modeling of the human SCOT monomer, and characterization of three pathogenic mutations, Genomics 68 (2000) 144–151.

[4] M. Cornblath, R.L. Gingell, G.A. Fleming, J.T. Tildon, A.T. Leffler, R.A. Wapnir, A new syndrome of ketoacidosis in infancy, J. Pediatr. 79 (1971) 413–418.

[5] J.T. Tildon, M. Cornblath, Succinyl-CoA: 3-ketoacid CoA-transferase deficiency. A cause for ketoacidosis in infancy, J. Clin. Invest. 51 (1972) 493–498.

[6] C. Perez-Cerda, B. Merinero, P. Sanz, A. Jimenez, C. Hernandez, M.J. Garcia, M. Ugarte, A new case of succinyl-CoA: acetoacetate transferase deficiency, J. Inherit. Metab. Dis. 15 (1992) 371–373.

[7] H. Sakazaki, K. Hirayama, S. Murakami, S. Yonezawa, H. Shintaku, Y. Sawada, T. Fukao, H. Watanabe, T. Orii, G. Isshiki, A new Japanese case of succinyl-CoA: 3-ketoacid CoA-transferase deficiency, J. Inherit. Metab. Dis. 18 (1995) 323–325.

[8] C.J. Pretorius, G.G. Loy Son, F. Bonnici, E.H. Harley, Two siblings with episodic ketoacidosis and decreased activity of succinyl-CoA:3-ketoacid CoA-transferase in cultured fibroblasts, J. Inherit. Metab. Dis. 19 (1996) 296–300.

[9] T. Fukao, X.Q. Song, H. Watanabe, K. Hirayama, H. Sakazaki, H. Shintaku, M. Imanaka, T. Orii, N. Kondo, Prenatal diagnosis of succinyl-coenzyme A:3-ketoacid coenzyme A transferase deficiency, Prenat. Diagn. 16 (1996) 471–474.

[10] K.E. Niezen-Koning, R.J. Wanders, J.P. Ruiter, L. Ijlst, G. Visser, W.C. Reitsma-Bierens, H.S. Heymans, D.J. Reijngoud, G.P. Smit, Succinyl-CoA:acetoacetate transferase deficiency: identification of a new patient with a neonatal onset and review of the literature, Eur. J. Pediatr. 156 (1997) 870–873.

[11] M.O. Rolland, N. Guffon, G. Mandon, P. Divry, Succinyl-CoA:acetoacetate transferase deficiency. Identification of a new case; prenatal exclusion in three further pregnancies, J. Inherit. Metab. Dis. 21 (1998) 687–688.

[12] S.E. Snyderman, C. Sansaricq, B. Middleton, Succinyl-CoA:3-ketoacid CoA-transferase deficiency, Pediatrics 101 (1998) 709–711.

[13] X.Q. Song, T. Fukao, H. Watanabe, H. Shintaku, K. Hirayama, S. Kassovska-Bratinova, N. Kondo, G.A. Mitchell, Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency: two pathogenic mutations, V133E and C456F, in Japanese siblings, Hum. Mutat. 12 (1998) 83–88.

[14] I. Baric, V. Sarnavka, K. Fumic, M. Maradin, D. Begovic, J.P. Ruiter, R.J. Wanders, A new case of succinyl-CoA:acetoacetate transferase deficiency: favourable course despite very low residual activity, J. Inherit. Metab. Dis. 24 (2001) 81–82.

[15] G.T. Berry, T. Fukao, G.A. Mitchell, A. Mazur, M. Ciafre, J. Gibson, N. Kondo, M.J. Palmieri, Neonatal hypoglycaemia in severe succinyl-CoA: 3-oxoacid CoA-transferase deficiency, J. Inherit. Metab. Dis. 24 (2001) 587–595.

[16] N. Longo, T. Fukao, R. Singh, M. Pasquali, R.G. Barrios, N. Kondo, K.M. Gibson, Succinyl-CoA:3-ketoacid transferase (SCOT) deficiency in a new patient homozygous for an R217X mutation, J. Inherit. Metab. Dis. 27 (2004) 691–692.

[17] T. Fukao, H. Shintaku, R. Kusubae, G.X. Zhang, K. Nakamura, M. Kondo, N. Kondo, Patients homozygous for the T435N mutation of succinyl-CoA:3-ketoacid CoA transferase (SCOT) do not show permanent ketosis, Pediatr. Res. 56 (2004) 858–863.

[18] T. Fukao, S. Sakurai, M.O. Rolland, M.T. Zabot, A. Schulze, K. Yamada, N. Kondo, A 6-bp deletion at the splice donor site of the first intron resulted in aberrant splicing using a cryptic splice site within exon 1 in a patient with succinyl-CoA: 3-ketoacid CoA transferase (SCOT) deficiency, Mol. Genet. Metab. 89 (2006) 280–282.

[19] K. Yamada, T. Fukao, G. Zhang, S. Sakurai, J.P. Ruiter, R.J. Wanders, N. Kondo, Single-base substitution at the last nucleotide of exon 6 (c.671G>A), resulting in the skipping of exon 6, and exons 6 and 7 in human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene, Mol. Genet. Metab. 90 (2007) 291–297.

[20] T. Fukao, P. Kursula, E.P. Owen, N. Kondo, Identification and characterization of a temperature-sensitive R268H mutation in the human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene, Mol. Genet. Metab. 92 (2007) 216–221.

[21] S. Merron, R. Akhtar, Management and communication problems in a patient with succinyl-CoA transferase deficiency in pregnancy and labour, Int. J. Obstet. Anesth. 18 (2009) 280–283.

[22] T. Fukao, T. Ishii, N. Amano, P. Kursula, M. Takayanagi, K. Murase, N. Sakaguchi, N. Kondo, T. Hasegawa, A neonatal-onset succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patient with T435N and c.658–666dupAACGTGATT p. N220_I222dup mutations in the OXCT1 gene, J. Inherit. Metab. Dis. (2010).

[23] T. Fukao, X.Q. Song, G.A. Mitchell, S. Yamaguchi, K. Sukegawa, T. Orii, N. Kondo, Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases, Pediatr. Res. 42 (1997) 498–502.

[24] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.

[25] X.Q. Song, T. Fukao, S. Yamaguchi, S. Miyazawa, T. Hashimoto, T. Orii, Molecular cloning and nucleotide sequence of complementary DNA for human hepatic cytosolic acetoacetyl-coenzyme A thiolase, Biochem. Biophys. Res. Commun. 201 (1994) 478–485.

[26] X.Q. Song, T. Fukao, G.A. Mitchell, S. Kassovska-Bratinova, M. Ugarte, R.J. Wanders, K. Hirayama, H. Shintaku, P. Churchill, H. Watanabe, T. Orii, N. Kondo, Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT): development of an antibody to human SCOT and diagnostic use in hereditary SCOT deficiency, Biochim. Biophys. Acta 1360 (1997) 151–156.

[27] P.D. Adams, P.V. Afonine, G. Bunkoczi, V.B. Chen, I.W. Davis, N. Echols, J.J. Headd, L. W. Hung, G.J. Kapral, R.W. Grosse-Kunstleve, A.J. McCoy, N.W. Moriarty, R. Oeffner, R.J. Read, D.C. Richardson, J.S. Richardson, T.C. Terwilliger, P.H. Zwart, PHENIX: a comprehensive Python-based system for macromolecular structure solution, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 213–221.

[28] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2126–2132.

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Differences between Human and Rodent Pancreatic Islets

LOW PYRUVATE CARBOXYLASE, ATP CITRATE LYASE, AND PYRUVATE CARBOXYLATION AND HIGH GLUCOSE-STIMULATED ACETOACETATE IN HUMAN PANCREATIC ISLETS[*][S]

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Anaplerosis, the net synthesis in mitochondria of citric acid cycle intermediates, and cataplerosis, their export to the cytosol, have been shown to be important for insulin secretion in rodent beta cells. However, human islets may be different. We observed that the enzyme activity, protein level, and relative mRNA level of the key anaplerotic enzyme pyruvate carboxylase (PC) were 80–90% lower in human pancreatic islets compared with islets of rats and mice and the rat insulinoma cell line INS-1 832/13. Activity and protein of ATP citrate lyase, which uses anaplerotic products in the cytosol, were 60–75% lower in human islets than in rodent islets or the cell line. In line with the lower PC, the percentage of glucose-derived pyruvate that entered mitochondrial metabolism via carboxylation in human islets was only 20–30% that in rat islets. This suggests human islets depend less on pyruvate carboxylation than rodent models that were used to establish the role of PC in insulin secretion. Human islets possessed high levels of succinyl-CoA:3-ketoacid-CoA transferase, an enzyme that forms acetoacetate in the mitochondria, and acetoacetyl-CoA synthetase, which uses acetoacetate to form acyl-CoAs in the cytosol. Glucose-stimulated human islets released insulin similarly to rat islets but formed much more acetoacetate. β-Hydroxybutyrate augmented insulin secretion in human islets. This information supports previous data that indicate beta cells can use a pathway involving succinyl-CoA:3-ketoacid-CoA transferase and acetoacetyl-CoA synthetase to synthesize and use acetoacetate and suggests human islets may use this pathway more than PC and citrate to form cytosolic acyl-CoAs.

Understanding the enzymatic makeup of human pancreatic islets is fundamental to developing strategies for designing artificial beta cells and beta cells differentiated from stem cells as treatments for type 1 diabetes, as well as modulating beta cell metabolism for the treatment of type 2 diabetes. Until recently, most of the information about normal insulin secretion came from studies of rodent islets or clonal cell lines. Although a recent study showed human pancreatic islets respond to insulin secretagogues similarly to rodent islets (1), what is still unknown is whether the use of intracellular pathways of secretagogue metabolism is the same in human islets as in rodent islets and cell lines. During the last few years, human islet preparations from human donors have become more readily available to researchers. By studying the levels of enzymes, the functional units of metabolism, the recent abundant supply of human islets has enabled our laboratory to discover clues suggesting differences in metabolic pathways between pancreatic islets of humans and rodents that have implications for better understanding normal human beta cell physiology.

Anaplerosis, the biosynthesis of citric acid cycle intermediates (2), is widely believed to be important for insulin secretion (3). Pyruvate carboxylase (PC)[2] is the key anaplerotic enzyme in this process and plays a central role in insulin secretion in the pancreatic beta cell of rodents and clonal insulin cell lines (3–8). In rat pancreatic islets, the level of PC is as high as in liver and kidney (4, 9–11), two organs in which PC plays a role in gluconeogenesis. In islets, which do not seem to require gluconeogenesis (12, 13), PC is concentrated in the beta cell (11). Our laboratory (4, 14–16) and, subsequently, Kahn *et al.* (17) previously showed that the rate of carboxylation of glucose-derived pyruvate catalyzed by PC is very high in rat pancreatic islets and equal to the rate of decarboxylation of glucose-derived pyruvate catalyzed by the pyruvate dehydrogenase complex. Lu *et al.* (18) showed that the rate of pyruvate cycling through PC is proportional to the capacity for glucose-stimulated insulin release from various INS-1 cell lines, and other studies have also found evidence for cycling of pyruvate through PC in rodent clonal beta cell lines (19, 20) and mouse pancreatic islets (21). More recently, we used RNAi knockdown technology to produce a series of cell lines derived from the rat insulinoma cell line INS-1 832/13 that expressed a range of PC levels. In these cell lines insulin release stimulated by glucose, as well as other metabolizable insulin secretagogues, was inhibited in proportion to the severity of PC knockdown (22). Severe knockdown

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2, Fig. 1, and Enzyme Assays.

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[2] The abbreviations used are: PC, pyruvate carboxylase; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; mGPD, mitochondrial glycerol phosphate dehydrogenase; PCC, propionyl-CoA carboxylase; PDC, pyruvate dehydrogenase complex; SCOT, succinyl-CoA:3-ketoacid-CoA transferase; BMI, body mass index.

Citrate and Acetoacetate Pathways in Human Islets

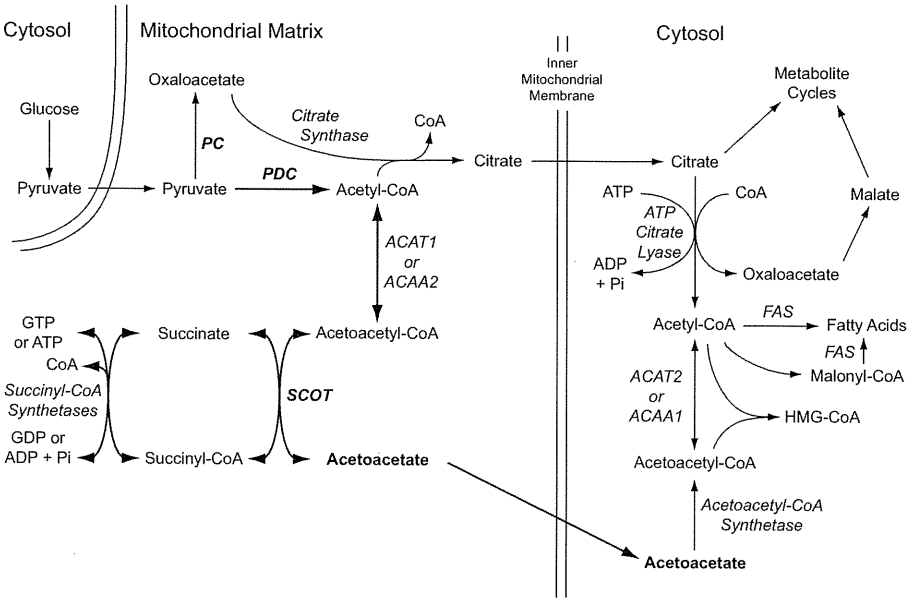

FIGURE 1. **Pathways of glucose-derived pyruvate via the pyruvate dehydrogenase complex and SCOT to produce acetoacetate and via pyruvate carboxylase and ATP citrate lyase to produce malate and citrate in mitochondria for export to the cytosol in the pancreatic beta cell.** The SCOT pathway is shown with *thicker arrows*. Abbreviations used are as follows: *ACAA1* or *ACAA2*, acetyl-CoA acyltransferase 1 or 2; *ACAT1* or *ACAT2*, acetyl-CoA acetyltransferase 1 or 2; *FAS*, fatty-acid synthase.

of PC caused a metabolite crossover point in glucose-stimulated cells, with increased pyruvate plus lactate and decreased malate and citrate consistent with a block at the PC reaction (22), further supporting a role for PC in insulin secretion. Another group also found that knockdown of PC inhibits insulin release in rat pancreatic islets (23), and yet another group found that moderate knockdown of PC in INS-1 cells and rat pancreatic islets did not inhibit insulin release possibly due to a compensatory increase in acetyl-CoA that may have activated PC as judged from an increase in acetylcarnitine, a surrogate for acetyl-CoA levels (24). The relatively high level of PC in rodent islets and beta cell lines and the other studies mentioned above unequivocally show that PC is very important for normal insulin secretion in these cells.

In view of the above information that established a role for PC in insulin secretion in rodent islets and clonal beta cell lines, we were surprised when, as a by-product of a very small study in which we compared gene expression and activities of various metabolic enzymes in islets from five nondiabetic humans to islets from five patients with type 2 diabetes (25), we noticed that PC mRNA and enzyme activity appeared to be much lower in islets from nondiabetic humans than we have recently seen in our separate unrelated studies of islets from nondiabetic rats or in clonal beta cell lines. Because of the important role of PC in insulin secretion in rodent beta cells and the preliminary suggestion that the level of PC might be low in human islets, we conducted a larger study focused on PC, as well as other enzymes intended to be controls, in islets from nondiabetic humans. We performed head-to-head comparisons of activities and/or protein levels of PC and other enzymes in human islets to their levels in islets of the rat and/or the mouse and the rat insulinoma-derived INS-1 832/13 cell line. In addition, we compared the relative rates of carboxylation and decarboxylation of pyruvate derived from glucose in intact human islets to these rates in intact rat pancreatic islets where the rate of pyruvate carboxylation is known to be high (14–17).

Our recent studies in rat pancreatic islets and INS-1 832/13 cells (26–29) suggest that beta cells have a pathway that is alternative to a pathway that uses PC and ATP citrate lyase to export carbon precursors of short chain acyl-CoAs to the cytosol. In this pathway, acetoacetate formed in the succinyl-CoA:3-keto-acid-CoA transferase (SCOT) reaction in mitochondria is exported to the cytosol where it can be converted to short chain acyl-CoAs and lipid by a series of reactions that begins with acetoacetyl-CoA synthetase (26–29) (Fig. 1). In the study reported here, the levels of SCOT, acetoacetyl-CoA synthetase, and ATP citrate lyase were among the enzymes we quantified in human islets and compared their levels in interassay measurements with those in rodent islets and the INS-1 832/13 cell line. We also compared glucose-stimulated levels of insulin release and acetoacetate in intact human islets and rat islets, and we studied the ability of β-hydroxybutyrate, the redox partner of acetoacetate, to potentiate glucose-stimulated insulin release in human islets. In addition, we included numerous tests within the study to discern whether the results could be artifacts caused by human donor conditions or the procedure for the preparation of human islets. The results suggest that, although metabolic pathways that require PC and ATP citrate lyase are important in rodent islets, human islets may preferentially use pathway(s) that require SCOT and acetoacetyl-CoA synthetase for stimulating insulin secretion.

EXPERIMENTAL PROCEDURES

Materials—Human pancreatic islets used in this study were from Washington University, St. Louis (n = 7 donors), Prodo Laboratories Inc., the Sharp-Lacy Research Institute, Irvine, CA (n = 16 donors), Massachusetts General Hospital (n = 6 donors), and City of Hope, Irvine, CA (n = 1 donor) sponsored

by the Juvenile Diabetes Research Foundation. Islets from the University of Wisconsin Islet Cell Resource Center, Madison (n = 9 donors), the University of Miami (n = 6 donors), the University of Pennsylvania, Philadelphia (n = 3 donors), and the University of Alabama, Tuscaloosa (n = 1 donor), were sponsored by the Islet Cell Research Consortium; and islets from the University of Southern California (n = 1 donor) were sponsored by the Integrated Islet Distribution Program. Two additional islet preparations were from Claes-Goran Ostenson at the Karolinska Institutet, Stockholm, Sweden. Human islet preparations are identified by a letter and/or a number in the legends of several figures. The significance of identifying the samples was simply to show that preparations from many different donors were studied.

Sprague-Dawley rats and all mice strains were from Harlan Laboratories, Madison, WI. The INS-1 832/13 cell line was from Chris Newgard. Diazomethane was generated *in situ* from (trimethylsilyl)diazomethane (Aldrich). A 10-fold excess was used to methylate [1,4-^{14}C]succinic acid and [2,3-^{14}C]succinic acid as described previously (14, 15). The reaction was performed in ethyl ether at 4 °C, and the product was evaporated to dryness and dissolved in water. With the exception of 1-^{14}C]pyruvate, which was from PerkinElmer Life Sciences, and was used in the assay of pyruvate dehydrogenase complex activity, all radioisotopically labeled chemicals were from American Radiolabeled Chemicals, Inc., St. Louis. Antibodies to human SCOT and ACAT1 and ACAT2 were from Dr. Toshiyuki Fukao. Anti-cow glutamate dehydrogenase antibody (catalogue number ab34786) and anti-human pyruvate dehydrogenase complex E1α subunit antibody (catalogue number ab97352) were from Abcam. Anti-human ATP citrate lyase antibody (catalogue number 1699-1) was from Epitomics. Anti-human fatty-acid synthase (catalogue number sc-20140) was from Santa Cruz Biotechnology, Inc. An antibody to human pyruvate dehydrogenase complex E2 protein was the IgG fraction from a pool of 50 primary biliary cirrhosis patients that was affinity-purified on a bovine pyruvate dehydrogenase column, and it was generously provided by Jeremy M. Palmer. The antibody also reacted with the E2 component of the α-ketoglutarate dehydrogenase complex. The antibody to rat acetoacetyl-CoA synthetase was from Tetsuya Fukui. Anti-β-actin and other chemicals in the highest purity available were from Sigma.

Islet Handling and Subcellular Fractionation—Human islets received in 2008–2011 from all sources were shipped in tissue culture medium. After the islets were received in our laboratory, they were maintained from 2 to 24 h in the medium in which they were shipped (CMRL 1066 tissue culture medium (or PIM when islets were from Prodo Laboratories)) and occasionally in RPMI 1640 medium modified to contain 5 mM glucose before use. Alternatively, islet pellets or whole-cell homogenates were prepared, and these were stored frozen until use. Human islets shipped from St. Louis in 1998 (n = 4) and from Stockholm in 2004–2008 (n = 2) were snap-frozen as islet pellets after isolation and shipped on dry ice.

For dilution to the optimal concentrations of enzyme protein for subsequent use in assays of enzyme activity and Western analysis, it was practical to homogenize human islets at a concentration of ~7,000 islets/ml in KMSH (220 mM mannitol, 70 mM sucrose, and 5 mM potassium Hepes buffer, pH 7.5) containing 1 mM dithiothreitol (4, 25, 27) to produce a homogenate with about 2 mg of whole-cell protein/ml of homogenate, which was diluted as needed at the time of use. This concentration of protein in a whole-cell homogenate was about the minimum required for optimal preservation of the activities of most of the enzymes studied. For the first 13 human islet samples received, a protease inhibitor mixture (ThermoScientific product 78415) was included in the homogenization solution. Subsequently, it was not included because its use produced no discernible difference in enzyme activities. When the activity of the pyruvate dehydrogenase complex was to be measured, the homogenizing solution contained 10 mM $MgCl_2$ and 1 mM dichloroacetate (30) in addition to the ingredients described above. Rat and mouse islets were isolated as described previously (4, 14–16) and homogenized in the KMSH/dithiothreitol solution described above, except the concentration of islets was about ~3,600 islets/ml of homogenizing solution. Some of the rat islet preparations were maintained in RPMI 1640 medium modified to contain 5 mM glucose for 24 h or as frozen homogenates or as islet pellets before use to produce control samples maintained under conditions similar to the human islets.

Enzyme Assays—Because the emphasis of the study was on PC, PC enzyme activity was measured first and usually in a fresh homogenate of human islets that had either not been frozen at all or was stored frozen for no more than 1 day. The activities of other enzymes were measured as soon as practical after PC was measured and in enough samples to discern whether their levels were different between the human islets and control islets or the INS-1 832/13 cell line. The numbers of human islets received in each shipment precluded the measurement of activities of all enzymes especially when the islets were also used for studies of islet metabolism. Activities of all enzymes were measured under V_{max} conditions. Pyruvate carboxylase (22), propionyl-CoA carboxylase (22), pyruvate dehydrogenase complex (13), mitochondrial glycerol phosphate dehydrogenase (31), and glutamate dehydrogenase (22) activities were measured in the whole-cell homogenates as described previously. Activities of ATP citrate lyase (24), malic enzyme (14, 32, 33), NADP isocitrate dehydrogenase (33, 34), malate dehydrogenase (35), and aspartate aminotransferase (35) were measured as described previously in a supernatant fraction of cells prepared so as to contain cytosol proteins and mitochondrial matrix proteins but not mitochondrial membrane proteins (the supernatant fraction from centrifuging the frozen-thawed whole-cell homogenate at 20,800 × g for 20 min) (22, 26). The concentrations of ingredients of the enzyme reaction mixtures and conditions of the more relevant enzyme assays are briefly described in the supplemental material.

Western Blot Analysis—Immunoblotting (26, 36) and probing of blots with streptavidin (4) were performed as described previously. Proteins were separated by SDS-PAGE, and after transfer to nitrocellulose, the membrane was blocked with a mixture of 10 mM Tris buffer, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (TBST), and 5% nonfat powdered dry milk and probed with a "first" antibody. The first antibody bound to a protein band was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo Scientific) used at 1:13,000.

The signal was detected using the Immobilon Western chemiluminescent HRP developer (Millipore). As a control for equal protein loading, the blot was stripped with Restore Western blot stripping buffer (Thermo Scientific) and reprobed with a polyclonal antiserum to β-actin diluted in TBST and 3% bovine serum albumin. For detecting biotin-containing proteins, PC and PCC, the nitrocellulose membrane was blocked with TBST buffer containing 2% gelatin. The streptavidin-horseradish peroxidase conjugate antibody (RPN1231, PerkinElmer Life Sciences) was used at 1:20,000 (diluted in TBST + 2% gelatin). The signal was detected using the West Pico CL kit (Pierce catalogue number 34077). To estimate relative densities of the protein bands captured on x-ray films exposed to the immunoblots, the areas and densities of the bands were measured with a Bio-Rad Chemidoc XRS Imaging System and calculated with Quantity One software.

Protein Concentration—The protein concentrations of whole-cell homogenates and supernatant fractions used for measurements of enzyme activity and immunoblot analysis were measured by the Bradford method. The amount of protein in pellets of intact islets from metabolite measurements was measured by the Lowry method after the protein precipitates were washed with 10% trichloroacetic acid.

Pyruvate Carboxylation Measured by $^{14}CO_2$ Ratios—The $^{14}CO_2$ ratios method was used to compare the fractions of glucose-derived pyruvate that enter mitochondrial metabolism by carboxylation and decarboxylation in human pancreatic islets *versus* in rat pancreatic islets. The $^{14}CO_2$ ratios method was developed by Kelleher and Bryan, III (37), and its use in rat pancreatic islets is described in Refs. 14–16. Briefly, the rate of $^{14}CO_2$ produced from the metabolism of [2-^{14}C]pyruvate is divided by the rate of $^{14}CO_2$ produced from [3-^{14}C]pyruvate to obtain a pyruvate $^{14}CO_2$ ratio. The rate of $^{14}CO_2$ produced from metabolism of [1-^{14}C]acetate is divided by the rate of $^{14}CO_2$ produced from [2-^{14}C]acetate to obtain an acetate $^{14}CO_2$ ratio. The pyruvate and acetate $^{14}CO_2$ ratios were then used in a formula to calculate the fractions of pyruvate that enter the citric acid cycle via pyruvate carboxylation catalyzed by pyruvate carboxylase and via decarboxylation catalyzed by the pyruvate dehydrogenase complex. As was done previously (14–16), we used glucose labeled with ^{14}C at positions 2 and 6, which, after metabolism via the glycolytic pathway, became pyruvate-labeled at positions 2 and 3, respectively, to obtain the pyruvate $^{14}CO_2$ ratio. We used dimethyl succinate labeled at the 1 plus 4 carbons and the 2 plus 3 carbons of the succinate part of the molecule, which can substitute for acetate labeled at positions 1 and 2, respectively, to obtain the acetate $^{14}CO_2$ ratio (14–16).

The fraction of pyruvate carboxylated was calculated according to Ref. 37, Equation 4, which is twice the difference of the acetate and pyruvate ratios divided by the following three terms: 1 + the pyruvate ratio, the acetate ratio − 1, and 2 − F, where F is the ratio of randomized to nonrandomized carbon in the reactions oxaloacetate to malate to fumarate and back to oxaloacetate. The values of F equal to 0.8 and 1.0 were used in this equation which assumes, respectively, almost complete and complete randomization of carbon in these metabolites. Because the activities of the enzymes catalyzing these reactions, fumarase[3] and malate dehydrogenase (Table 4) (25, 38), are very high in islets, this is a reasonable assumption.

Human or rat pancreatic islets (100/test tube) were incubated for 90 min in Krebs-Ringer bicarbonate Hepes buffer, pH 7.3, containing 0.5% fatty acid-free bovine serum albumin, in the presence of [2-^{14}C]glucose and [6-^{14}C]glucose (16.7 mM) (specific radioactivity 0.2 mCi ^{14}C/mmol glucose) and dimethyl [1,4-^{14}C]succinate and dimethyl [2,3-^{14}C]succinate (10 mM) (specific radioactivity 0.2 mCi ^{14}C/mmol dimethyl succinate).

Metabolite Measurements—Acetoacetate and malate levels were measured by alkali-enhanced fluorescence as described previously (38–40).

Insulin Release—Insulin release was studied as described previously (13, 31, 35) with the same average size and similar number (confirmed by islet protein measurements) of rat or human islets per vial incubated for 1 h in the presence of Krebs-Ringer bicarbonate buffer modified to contain 10 mM Hepes buffer, pH 7.3, and 0.5% fatty acid-free bovine serum albumin. Insulin was measured by a radioimmunoassay with human insulin or rat insulin as a standard. Rat islets used for insulin release were from 2- to 2.5-month-old male and female animals weighing 225–250 g. The average BMI and age of the human islet donors whose islets were used for insulin release are mentioned in Table 5.

Quantitative PCR—Tissues were homogenized with a Qiashredder (Qiagen) (islets) or using a Potter-Elvehjem homogenizer (liver), and RNA was prepared using the RNeasy mini kit (product number 74104, Qiagen). On-column DNase digestion was performed using the Qiagen RNase-free DNase set. cDNA was made with randomized primers with the Retroscript kit (AM1710) (Applied Biosystems). Quantitative PCR was performed on a MyIQ real time detection system (Bio-Rad) with SYBR Premix Ex Taq (RR041Q) (Takara). mRNAs encoding other metabolic enzymes were measured as internal controls for PC mRNA measurements. Nucleotide sequences of primers used are shown in supplemental Table 2. Human islet RNAs were compared with RNAs from a liver of a 51-year-old male (Clontech, catalogue number 636531) and a surgical specimen from an adult human liver (of unknown gender and age due to privacy protection) from the University of Wisconsin Hospital. RNA was isolated from islets of human donors whose average BMI and age are shown in Table 5. RNA from rat islets was from 8- to 10-week-old male rats and RNA from rat liver was from two 8- to 10-week-old male rats and two 11-month-old female rats.

Human PC mRNA was measured with two primer sets (PC1 (789–864) and PC2 (2042–2131)). The relative level of the PC mRNA was divided by the relative levels of other metabolic enzyme mRNAs, and these ratios were expressed as a percent of the same ratios in liver with the liver values expressed as 100%. The ratios of the human islets *versus* liver were compared with the same ratios from the rat. The integrity of the RNA samples and their concentration were measured with the Agilent 2100 Bioanalyzer using RNA6000 NanoChips at the University of Wisconsin Biotechnology Center. Integrity numbers, where 10 is the highest and integrity numbers of above 6 indicate RNA suitable for accurate PCR measurements (41), were as follows:

[3] M. J. MacDonald, unpublished observations.

TABLE 1

Lower PC enzyme activity in pancreatic islets than rat islets or INS-1 832/13 cells

Enzyme activities in whole-cell homogenates of human islets were compared with activities in homogenates of either rat islets or INS-1 832/13 cells measured in the same assay batch. PC was also measured in islets of older rats as a control for aging. Results are the mean ± S.E. with the number of observations in parentheses. The activity of propionyl-CoA carboxylase (PCC), an enzyme that catalyzes a reaction similar to PC, is shown as an additional control. The mean ± S.E. BMI and age of the human islet donors whose islets were used for these measurements were 28.9 ± 1.9 kg/m^2 and 51.3 ± 2.6 years.

Islet source	PC activity	PCC activity
	nmol of CO_2 fixed per min/mg of protein	*nmol of CO_2 fixed per min/mg of protein*
Human islets	6.3 ± 0.8 (23)[a]	25.3 ± 4.1 (6)
Same assay control, rat (age 2 months) islets	45.5 ± 4.4 (7)	29.0 ± 1.6 (7)
Same assay control, INS-1 832/13 cells	44.8 ± 2.5 (15)	1.6 ± 0.2 (9)
Rat (age 11 months) islets	59.8 ± 3.3 (6)	

[a] $p < 0.001$ *versus* rat islet or INS-1 832/12 cell PC values.

human islets (9.1, 8.5, 9.1, 8.5, 9.0, 7.4, 8.7, 9.2, and 9.0), human liver (7.4 and 8.0), rat islets (5.5, 7.1, 6.6, and not done), and rat liver (9.1, 9.0, 7.4, and not done). In addition, the comparison of PC mRNA with other mRNAs in the same tissue also serves as a control for RNA quality.

Statistical Significance—Statistical significance of differences was assessed by Student's unpaired *t* test.

RESULTS

Characteristics of Human Islet Donors and Islets—Human islet preparations from 52 donors were studied. Ages of the donors ranged from 19 to 74 years (mean age ± S.E. = 44.9 ± 2.1 years, median age = 46 years). Twenty four of the 48 donors for whom the gender was known were female and 24 were male. BMI when recorded (n = 47) ranged from 20.1 to 51.2 kg/m^2 (mean ± S.E., 29.4 ± 1.1 kg/m^2) (median = 28.6 kg/m^2). The BMIs of 15 donors were between 20 and 26 kg/m^2; 21 were between 26 and 32 kg/m^2; 5 were between 33 and 36 kg/m^2; 5 were between 37 and 44 kg/m^2, and 1 was equal to 51.2 kg/m^2. Average BMIs and ages of donors whose islets were used for various measurements are shown in the corresponding tables or figures. The purity of 29 islet preparations was rated as 90–99%, 12 as 80–89%, 6 as 76–79%, 4 as 70%, and 1 as 68% by the laboratories that isolated the islets. The viability was described as 90–98% for all but four islet preparations that were described as 82–87% viable.

Influences of Protein Concentration on Enzyme Activity—Because either too little or too much protein in the enzyme reaction mixture may each lower the specific enzyme activity, optimal concentrations of protein in the enzyme reaction mixtures were determined for the PC assay and the other assays. The concentrations of protein and the volume of extract added and the final volumes of reaction mixture for the more relevant enzyme assays are listed in supplemental Table 1. Every PC and PCC enzyme reaction mixture contained 10 μl of 2 mg whole-cell protein/ml in a final volume of 50 μl.

Low PC Enzyme Activity in Human Islets—Measurements of PC enzyme activity were performed on the first 23 excellent quality samples of human islets received and always within an hour of homogenization of the islets or within an hour of the first thaw of a homogenate stored for 1 day or a shorter time period. Human islets received subsequently were used for $^{14}CO_2$ ratios, metabolite, and mRNA measurements and insulin secretion experiments (see below.).

In each PC assay, a rat islet sample that was maintained similarly to the human islet sample (in tissue culture, as a frozen islet pellet, or a briefly frozen fresh homogenate) and/or an INS-1 832/13 sample (usually stored frozen as a whole-cell homogenate for several days or weeks) was included as a control along with the human islet sample. Most of the human islet samples were maintained for 2–4 or 24 h in tissue culture medium (usually CMRL medium or PIM medium (which each contain 5 mM glucose)) after they were received, and the PC assay was performed immediately after the tissue culture and homogenization of the islets without freezing of the whole-cell homogenate. When human islets were received as frozen pellets without prior tissue culture, the PC assay was performed within an hour of thawing of the islet pellet (very few samples). Regardless of the conditions under the human islets were maintained prior to the PC assay, all of the PC values were similar and much lower than the values from rat islets and INS-1 832/13 cell samples maintained under similar conditions prior to the measurements of PC enzyme activity. Maintaining human islets in RPMI 1640 tissue culture medium with the glucose concentration adjusted to 5 mM (as is used to maintain rat islets in tissue culture in our laboratory) for 2–4 or 24 h did not produce PC enzyme activities that were different from those in islets maintained for any length of time in CMRL medium or PIM medium.

The PC enzyme activity of samples of islets from the 23 human donors studied for PC activity averaged 13 ± 2% of the same assay control values (6.3 ± 0.8 (means ± S.E.) *versus* ≅45 nmol of CO_2 fixed per min/mg of whole-cell protein) of the PC activity in rat islets and in INS-1 832/13 cells (Table 1). This tight clustering of human islet PC values around 13% of the control PC values, instead of being spread across a large range from equal to the values of rat islets to close to zero, suggests the low values are intrinsic to human islets rather than an artifact of handling of the islet preparations. The same-assay rat islet preparations were from 2- to 2.5-month-old rats. As an additional control for older age, PC activity was also measured in islets from 11-month-old rats. This PC value was even higher than the same-assay rat islet PC activity (Table 1).

On the possibility that various subtypes of islets possess superior characteristics that might be associated with higher PC activities, the islets were classified into various subcategories. None of these categories showed higher PC activities. These were handpicked islets (that might have been more pure) (PC activity = 6.9 ± 0.3 (3)), small human islets (small human islets have been reported to show higher glucose-stimulated insulin release *in vitro* and after transplantation in humans

TABLE 2

Lower PC enzyme activity in the highest quartile of human islet PC values compared with same assay rat islet or INS-1 832/13 cell PC values

PCC activity is shown as an additional control. PC and PCC enzyme activities in whole-cell homogenates are from Table 1 and are the mean ± S.E. with the number of preparations studied in parentheses.

	Enzyme activity
	nmol of CO_2 fixed per min/mg of protein
Highest quartile of human islet PC values	11.9 ± 1.1 (5)[a]
Highest eight human islet PC values	9.6 ± 1.0 (8)[a]
Same assay rat islet PC values	53.4 ± 2.2 (3)
Same assay INS-1 832/13 PC values	53.0 ± 3.3 (5)
PCC value of highest PC quartile human islets	26.9 ± 4.7 (5)
Same assay rat islet PCC values	30.6 ± 1.7 (4)

[a] $p < 0.001$ *versus* rat islet or INS-1 832/12 cell PC values.

(42)) (PC activity = 5.7 and 7.3 (2)), and islets maintained for 24 h in the RPMI 1640 tissue culture medium modified to contain 5 mM glucose (the same medium used to maintain the rat islets) (PC activity = 4.8 ± 1.2 (3)), as well as entire human islet preparations (PC activity = 6.5 ± 0.8 (18)), which should have been a mixture representative of all the islets of the pancreas (mean PC activity in nmol CO_2 fixed per min/mg of protein ± S.E. (*N*)).

Even when the very highest PC enzyme activities of the human islets were compared with the PC values of the rat islets and/or the INS-1 832/13 rat insulinoma cell line measured in the same assay batch, the human islet PC enzyme activities were still much lower than the rodent values. The average of the highest quartile ($n = 5$) of human islet PC values was only 22 ± 2.2%, and the average of the highest eight human islet PC values was 18 ± 1.8%, of the same-assay rat islet and INS-1 832/13 cell PC values (Table 2).

Low PC Protein in Human Islets—As a biotin-containing protein, PC can be detected with streptavidin that binds to biotin-containing proteins in Western blots. In agreement with the low PC enzyme activities, Western blot analysis with streptavidin showed that the PC protein was much lower in the samples of islets from human donors compared with similarly treated rat and mouse islets and INS-1 832/13 cells. This was the case whether the human and rodent islets were processed immediately after the islets were received, after tissue culture for 2 h to 1 day, and after islets were stored frozen as pellets at −20 °C for about 1 month or at −70 °C for up to 10 years (Figs. 2 and 3). The low PC in human islets cannot be explained by the human PC being more readily degraded during frozen storage because the PC protein in human liver was not low after frozen storage for 7 months (Fig. 3). A densitometric scan of the PC protein bands showed that the density of the human islet PC bands was 5 ± 1% (6) (mean ± S.E. (*N*)) of that of the rat and mouse PC bands (Fig. 3).

Propionyl-CoA Carboxylase Is Not Low in Human Islets—An excellent control for PC is propionyl-CoA carboxylase (PCC), which like PC is a biotin-containing enzyme and catalyzes a reaction similar to PC. The average PCC enzyme activity from all samples of human islets in which PCC was measured was similar to that in the rat islets (Table 1). (The PCC values in INS-1 832/13 cells were much lower than in human and rat islets.) In addition, the average of the same sample human islet

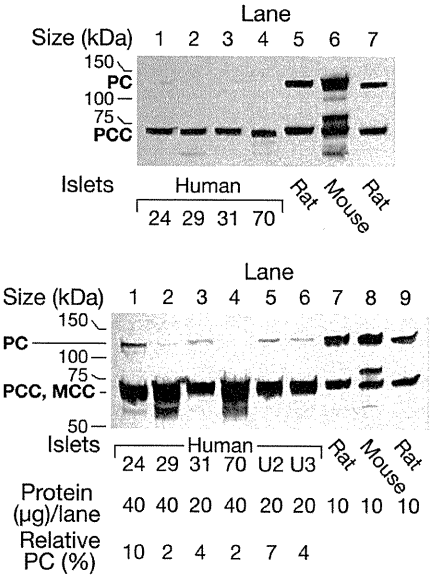

FIGURE 2. **Level of pyruvate carboxylase protein is much lower in human pancreatic islets than in mouse and rat islets.** The *upper panel* shows a streptavidin-probed blot of islets from four human individuals identified by *numbers* and islets from the rat and the mouse. Each preparation was snap-frozen immediately after isolation. All of these samples happen to have been stored frozen as islet pellets for 8–10 years before they were homogenized in a solution of KMSH containing 1 mM dithiothreitol and analyzed. There was 15-μg whole-cell proteins per lane. The band that migrates at ~130 kDa is PC and is not visible (*lanes 2* and *4*) or barely visible (*lanes 1* and *3*) in the islets from the humans. The band at ~72 kDa is the α-chain of propionyl-CoA carboxylase (*PCC*) (with or without methylcrotonyl-CoA carboxylase (*MCC*) that migrates close to the α-chain of PCC). The density of this band is relatively the same in lanes of the human and rodent islets. The *lower panel* shows the semiquantification of PC protein and is a streptavidin-probed blot in which larger amounts (20 or 40 μg) of whole-cell protein were added to the lanes containing human islet samples than were added to the lanes containing rodent islet samples. *Lanes 1–4* contained the human islets and *lanes 7* and *8* contained the rat and mouse islets shown in the *top panel*. *Lanes 5* and *6* contained human islet samples homogenized and boiled in SDS gel sample buffer on the day of receipt and analyzed 1 month and 1 week later, respectively; and *lane 9* contained rat islets stored as a frozen homogenate 3 months before analysis. Densitometric quantification of the PC band indicated that the amount of PC protein/μg islet cell protein was 2–10% of the average of the amounts of PC in the three rodent lanes.

PCC values in human islets with the highest quartile of PC values, when measured, was similar to the PCC values of rat pancreatic islets measured in the same assay batch (Table 2).

As a biotin-containing enzyme, PCC protein, like PC protein, can be visualized with streptavidin on Western blots. PCC protein was not low in human islets compared with rat and mouse islets (Fig. 2) and compared with rat liver and human liver (Fig. 3). This suggests that the lower PC values in human islets were specific for PC and not due to a generalized decreased expression or stability of biotin-containing carboxylases in human islets compared with islets of rats or mice.

Low ATP Citrate Lyase and Protein in Human Islets—The enzyme activity of ATP citrate lyase was 75 and 77% lower in human islets than in rat islets and the INS-1 832/13 line (Table 3), respectively, which was proportionately not as low as PC (Table 1).

The low ATP citrate lyase was confirmed with immunoblots with anti-ATP citrate lyase antibody that showed ATP citrate lyase protein was low in human islets relative to rat islets and INS-1 832/13 cells (Fig. 4). The average relative density per μg

of whole-cell protein/lane of the ATP citrate lyase bands was 42 ± 7% (4) of the same band of the rat islets (100 ± 10% (4)) (means ± S.E. (*N*)) ($p < 0.01$) (*lower panel* of Fig. 4).

Enzymes Not Decreased in Human Islets—Mitochondrial glycerol phosphate dehydrogenase (mGPD) is expressed at a very high level in beta cells of the pancreatic islets of rats (31, 43, 44), mice (45), and humans (Table 3) (25, 46, 47) relative to most tissues of the body. Thus, the level of mGPD serves as a good control for other islet mitochondrial enzymes. If human islets were less pure or of lower quality than rodent islets, the level of mGPD in human islets might be expected to be lower than in rodent islets. The level of mGPD in the human islets was as high as in the rat islets (Table 3) indicating human islets were as pure and of good quality as the rat islets. The level of mGPD in both human and rat islets was 33% lower than in INS-1 832/13 cells most likely because the INS-1 832/13 cells are pure beta cells, whereas islets contain non-beta endocrine cells, as well as connective tissue cells, in addition to beta cells.

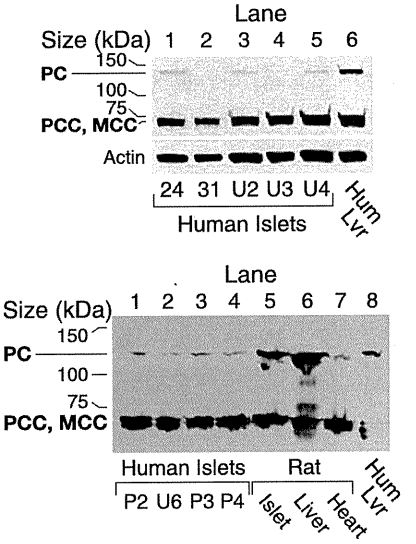

FIGURE 3. **Low level of PC in human islets cannot be explained by exquisite instability of the human PC protein because the PC protein is stable in human liver.** *Upper panel,* streptavidin-probed blot with 15 μg of cell protein/lane. Human liver (*Hum Lvr*) was stored frozen for 7 months before analysis. Human islets 24 and 31 were snap-frozen and stored 8–10 years, and islets U2 and U3 were cultured on 1 day and placed in gel loading buffer the same day and then analyzed at <4 weeks (*U2*) or <2 weeks (*U3*) or snap-frozen right after isolation and put in loading buffer the same day and analyzed 5 days later (*U4*). The density of the PCC/methylcrotonyl-CoA carboxylase (*MCC*) bands is relatively the same across the lanes. The membrane was stripped of streptavidin and reprobed with anti-β-actin antibody to show relatively equal loading of total cell protein across lanes. *Lower panel,* low PC protein in fresh cultured human pancreatic islets compared with rat islets, rat liver, or human liver. This panel shows a streptavidin-probed blot with 15 μg of whole-cell protein/lane, except for *lane 8* where the protein equaled 4 μg. Human islet samples in *lanes 1–4* were maintained in tissue culture medium for 2 or 24 h prior to homogenization. Rat heart is shown as a tissue in which the level of PC is low.

The activities of cytosolic malic enzyme (ME1) (EC 1.1.1.40), aspartate aminotransferase, NADP isocitrate dehydrogenase, glutamate dehydrogenase, the pyruvate dehydrogenase complex (PDC), and malate dehydrogenase in human islets were not significantly different from those in rat islets and/or the INS-1 832/13 cell line (Table 3). Immunoblots showed that the levels of PDC E2 protein and α-ketoglutarate dehydrogenase complex E2 protein (Fig. 5), as well as glutamate dehydrogenase protein and acetyl-CoA acetyltransferases 1 and 2 proteins (Fig. 5), were also not decreased in human islets. The activity of the pyruvate dehydrogenase complex was lower in INS-1 832/13 cells than in human and rat islets (Table 3), and this was confirmed with an immunoblot that showed the density of the PDC E1α protein, the rate-limiting enzyme of the PDC, in INS-1 832/13 cells averaged 38% that in human islets as judged by densitometry measurements (Fig. 5). The average human islet and INS-1 832/13 malate dehydrogenase activities were lower than the average rat islet malate dehydrogenase activity (Table 2). Although the measurements of PC and ATP citrate lyase activities were always made on the freshest possible samples, it was logistically necessary to measure activities of some of the enzymes in human islets after several days or weeks of frozen storage of islet homogenates. Because the malate dehydrogenase measurements in human islets fell into this category, the human islet malate dehydrogenase activity represents the minimal true value. Malate dehydrogenase activity is extremely high in any of the tissues compared with the activities of other enzymes (Table 3), such that differences in the average malate dehydrogenase activities may not cause significantly different rates of intermediary metabolism among the various tis-

TABLE 3

Activities of various enzymes in human islets compared with rat islets or INS-1 832/13 cells

Results are the mean ± S.E. of the number of samples from individual donors or batches of rat islets or individual plates of INS-1 832/13 cells studied and shown in parentheses. The mean ± S.E. BMI and age of the human islet donors whose islets were used for the ATP citrate lyase measurements were 32.4 ± 2.6 kg/m^2 and 48.1 ± 3.6 years.

Enzyme	Enzyme activity		
	Human islets	Rat islets	INS-1 832/13
		nmol product/min/mg protein	
ATP citrate lyase[a]	24 ± 1.9 (21)[d]	96 ± 9.0 (13)	104 ± 3.5 (31)
mGPD[c]	29 ± 2.2 (16)	28 ± 1.5 (7)	43 ± 2.8 (9)
Cytosolic ME (ME1)[a]	34 ± 3.0 (16)	32 ± 2.0 (6)	38 ± 4.2 (8)
Aspartate aminotransferase[a,b]	595 ± 32 (7)	778 ± 116 (10)	528 ± 32 (9)
NADP isocitrate dehydrogenase[a,b]	208 ± 20 (12)	235 ± 30 (6)	198 ± 19 (10)
Glutamate dehydrogenase[c]	96 ± 7.6 (8)	129 ± 25 (6)	99 ± 7.7 (10)
Malate dehydrogenase[a,b]	7000 ± 800 (7)	16,300 ± 3,300 (9)	6900 ± 900 (8)
Pyruvate dehydrogenase[c]	15.1 ± 1.7 (10)	14.2 ± 1.3 (8)	3.7 ± 0.2 (12)

[a] Enzyme rates are expressed per mg of protein in the whole-cell homogenate supernatant fraction. Homogenate supernatant fractions were prepared so that mitochondria would be ruptured.

[b] Thus, in the case of isoforms of the three enzymes that are present in both the mitochondrial matrix and the cytosol, the values shown represent the sum of both activities.

[c] Enzyme rates are expressed per mg of protein of whole-cell homogenate protein.

[d] p is < 0.001 *versus* same assay rat islet and INS-1 832/13 cell values.

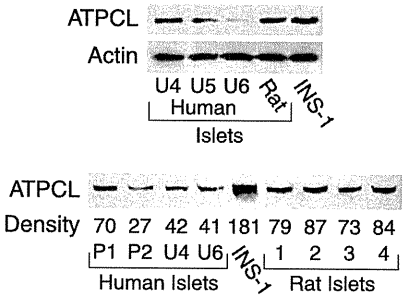

FIGURE 4. **Lower level of ATP citrate lyase protein in human islets than in rat islets.** *Top panel,* immunoblot with 15 μg of whole-cell protein in each lane. The actin bands show equal loading of protein across the lanes. *Lower panel,* immunoblot with 15 μg of whole-cell protein/lane in *1st* to *6th lanes. 7th* to *9th lanes* (from *left*) had 12, 11, and 9 μg of protein/lane, respectively. The relative densities of each ATP citrate lyase (*ATPCL*) band are shown at the *bottom* of the band without correction for the lower amounts of protein in the *7th* to *9th lanes* that contained rat islet protein.

sues. We recently showed that mitochondrial malic enzyme ME2 is present in human islets and its levels of enzyme activity and protein are similar to those in the islets of rats or mice or the INS-1 832/13 cell line (48).

Low Relative Rate of Pyruvate Carboxylation in Human Islets Versus Rat Islets—The $^{14}CO_2$ ratios method was used to compare the fractions of glucose-derived pyruvate that enter the citric acid cycle by carboxylation and decarboxylation in human pancreatic islets *versus* in rat pancreatic islets. If any pyruvate enters mitochondrial metabolism through PC, the pyruvate $^{14}CO_2$ ratio will be lower than the acetate $^{14}CO_2$ ratio, and if pyruvate is metabolized exclusively through the pyruvate dehydrogenase complex, the two ratios will be identical (14–16, 37). In view of the lower amount of amount of PC in human islets compared with rodent islets, the expected finding is a lower relative fraction of pyruvate carboxylation in human islets than in the rat islets, and this is what we observed. The acetate $^{14}CO_2$ ratio was much higher than the pyruvate $^{14}CO_2$ ratio in the rat islets, whereas in human islets the two ratios were very close to one another (Table 4). Thus, the data show that although both human and rat pancreatic islets metabolize pyruvate via both carboxylation and decarboxylation, the percentage of pyruvate carboxylated compared with the total pyruvate metabolized by carboxylation plus decarboxylation is 70–80% lower in human islets than in rat islets (46% *versus* 14% assuming $F = 0.8$ and 55% *versus* 17% assuming $F = 1$ of total (de)carboxylation (Table 4)).

Small human islets are reported to be superior with respect to insulin secretion after transplantation than human islets of average size (42). The fraction of pyruvate carboxylated compared with that decarboxylated was even lower in an additional two batches of human small islets studied (data not shown).

Islet PC mRNA Levels Relative to Liver PC mRNA Levels Are Much Lower in the Human—In the rat the activity and protein of PC in the islet are similar to those in the liver (Fig. 3) (4, 9–11). To see whether the low level of PC in human islets might be due to lower PC gene transcription than in rat islets, the ratio of PC mRNA to the mRNA levels that encode six other metabolic enzymes were determined and expressed relative to these same ratios in liver of the same species. Table 5 shows that, even though the integrities of the human islet and rat liver RNA

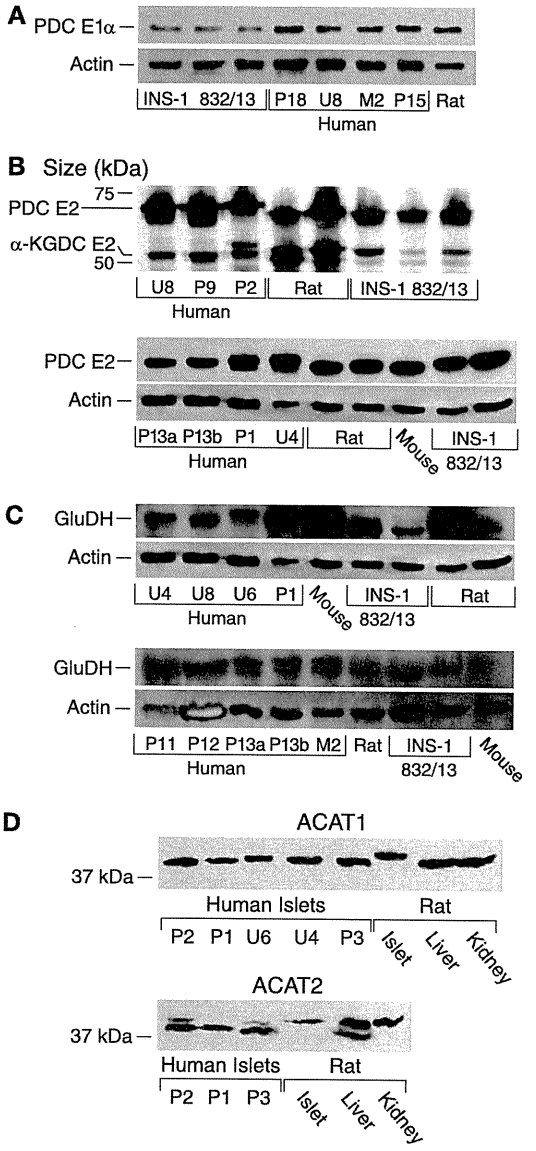

FIGURE 5. **Levels of pyruvate dehydrogenase complex E1α and E2 proteins, α-ketoglutarate dehydrogenase complex E2 protein, glutamate dehydrogenase protein, and acetyl-CoA acetyltransferases 1 and 2 proteins in human islets are comparable with or higher in human islets than in rat islets or INS-1 832/13 cells.** *A,* levels of PDC E1α protein in human and rat islets are higher than in INS-1832/13 cells. Immunoblot is shown with 15 μg of protein of whole-cell protein/lane probed with anti-PDC E1α antibody and stripped of antibody and reprobed with anti-β-actin antibody to show relative loading of protein across lanes. *B, top panel,* immunoblot with 20 μg of whole-cell protein/lane. It was probed with affinity-purified primary biliary cirrhosis (*PBC*) IgG at a 1:20,000 dilution that reacts against the PDC and α-ketoglutarate dehydrogenase complex (*KDC*) E2 proteins. *Lower panel,* immunoblot with 5 μg of whole-cell protein/lane probed with the primary biliary cirrhosis IgG at a dilution of 1:30,000 and reprobed with anti-β-actin antibody to indicate the relative total cell protein levels across the lanes. *C,* two immunoblots. Membranes were probed with anti-glutamate dehydrogenase (*GluDH*) antibody, stripped of antibody, and reprobed with anti-β-actin antibody to show relative loading of protein across lanes. *Lanes* in both panels contained 15 μg of whole-cell protein/lane except *3rd lane* from *left* in the *upper panel* contained 13 μg of protein/lane. *D, bottom panels,* immunoblots with 15 μg of whole-cell protein/lane (*ACAT1,* mitochondrial acetyltransferase; *ACAT2,* cytosolic acetyltransferase).

samples were superior to those of the rat islet RNA and human liver RNA samples (see under "Experimental Procedures"), which are combinations that could have produced higher rela-