Plasma and Skeletal Muscle Free Amino Acids in Type I, Insulin-treated Diabetic Subjects

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SUMMARY

Insulin-dependent diabetes mellitus (IDDM) induces plasma amino acid (AA) abnormalities, including low alanine and high branched-chain (BCAA). While insulin treatment restores plasma AA pattern, proline, methionine, valine, isoleucine, and total BCAA remain elevated in skeletal muscle intracellular water. This suggests that the restoration of plasma AA concentrations is not a satisfactory index of recovered AA metabolism in IDDM. DIABETES 1985; 34:812–15.

Disorders in amino acid (AA) metabolism are known in both human and experimental diabetes.[1-3] The main abnormalities are low alanine, serine, aspartic acid, glycine, threonine and high BCAA.[1,3] The limited ability of the diabetic subject to utilize glucose would result in increased use of AA for hepatic gluconeogenesis. On the other hand, lack of insulin would reduce their uptake by muscle after a meal and increase their release in the postabsorptive state. Since the liver has only limited capacity to degrade BCAA, low glucogenic AA and high BCAA plasma concentrations might be expected.[1,3-5]

These changes in circulating AA have been reported to be controlled in human diabetes by insulin treatment.[6] It has been suggested that plasma BCAA levels may be considered as a marker of short-term metabolic control in diabetes.[7] Insufficient information is available at this time on muscle intracellular concentration of AA in untreated human diabetes; animal studies have shown that several muscle free AAs, particularly BCAA, are high in experimental diabetes.[2]

The aim of this study was to evaluate whether insulin therapy is able to restore the altered intracellular AA pattern in human type I diabetes. To this purpose, we measured skeletal muscle and plasma free AA in conventionally treated type I diabetic subjects.

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MATERIALS AND METHODS

Eight nonobese, insulin-dependent diabetic subjects (6 men and 2 women) were studied. The age range was 32–49 yr, body weight was 84–107% of ideal body weight (IBW) according to Metropolitan Life Insurance tables, New York, 1959. Their dietary regimen was 250–300 g carbohydrate, 60–80 g protein, and 30–50 g fat per day. Conventional subcutaneous (s.c.) insulin therapy was performed in all patients. Regular insulin (Actrapid Novo) and lente insulin (Monotard Novo) were administered twice daily (20–50 U/24 h). The average duration of diabetes was 13 yr. The patients were informed of the significance of the test and possible side effects. None of them had evidence of hepatic or renal failure, according to physical and biochemical examination. Blood glucose levels, urinary glucose, and ketones in the 48-h period preceding the test were recorded. After an overnight fast, the patients received regular insulin (7 U, s.c.) 30 min before a standard breakfast; they were then not allowed to eat until 5 p.m. when the muscle biopsy was performed. At the same time, venous blood for AA and chloride determinations was drawn; arterial pH and HCO_3 were also evaluated.

Nine healthy volunteers (6 men and 3 women) in the same condition of postabsorptive state were examined as controls. Their ages ranged 43–60 yr and body weights corresponded to 88–109% of IBW.

Blood and urine glucose were measured by a glucose-oxidase method (Test Combination, Boehringer), urine ketones by Ace-test strips, and arterial pH and bicarbonate with a radiometer gas analyzer.

Muscle biopsy and analysis technique. Muscle biopsy of the quadriceps was carried out using the needle described by Bergström.[8-11] Each muscle sample, weighing 30–60 mg, was rapidly freed from fat, connective tissue, and blood, then divided into two fragments.

The first was immediately weighed and homogenized in a glass Potter-Evelhjem apparatus with 0.8–1.2 ml of 6% cold

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TABLE 1
Plasma amino acid concentrations (μM/kg plasma water, mean ± SEM) in eight insulin-treated diabetic and nine healthy subjects

	Control subjects (N = 9)	Diabetic subjects (N = 8)
Aspartic acid	16 ± 1	7 ± 1†
Threonine	119 ± 11	107 ± 7
Serine	123 ± 11	116 ± 7
Asparagine	52 ± 5	49 ± 9
Proline	208 ± 8	219 ± 19
Glycine	199 ± 19	222 ± 29
Alanine	295 ± 10	278 ± 20
Valine	215 ± 14	199 ± 9
Cystine	48 ± 5	60 ± 6
Methionine	22 ± 2	33 ± 7
Isoleucine	76 ± 4	77 ± 7
Leucine	132 ± 10	133 ± 10
Tyrosine	63 ± 7	53 ± 3
Phenylalanine	49 ± 5	59 ± 4
Ornithine	78 ± 6	39 ± 6†
Lysine	186 ± 11	156 ± 13
Histidine	92 ± 4	65 ± 3
Arginine	91 ± 8	103 ± 16
Taurine	73 ± 15	164 ± 11*
BCAA	423 ± 27	410 ± 64
Phenylalanine/tyrosine	0.80 ± 0.06	1.12 ± 0.06*

*$P < 0.005$.
†$P < 0.001$.

sulfosalicylic acid; plasma was precipitated with 3% cold sulfosalicylic acid. Supernatants from both plasma and muscle tissue were stored at −40°C until AA determination. AA were measured using the Carlo Erba 3A29 Automatic Aminoanalyzer with a one-column procedure and four lithium citrate buffers with pH from 2.78 to 5.42. Eluted AA were detected by photometry after ninhydrin reaction with reading at wavelengths of 570 and 440 nm. An external standard containing appropriate concentrations of 21 AA was analyzed every five samples. The size of each peak was measured using an integrator (Spectraphysic SP4000). The AA concentrations were calculated in reference to the standards and expressed as μmol/kg of muscle wet weight (AA_m) or per kg of plasma water (AA_{ECW}). It is well known that glutamine and glutamic acid are unstable under both storage and analysis conditions; similar behavior was observed for muscle aspartic acid. Finally, a good separation of asparagine and cystine peaks in muscle was achieved only in some subjects. Therefore, glutamine and glutamic acid are not included in the results, while aspartic acid, asparagine, and cystine values are reported only as plasma levels.

In the second muscle fragment, fresh weight was obtained by weighing at several time intervals and extrapolating to zero time.

Total muscle water (TW) was measured after drying for 2 h at 105°C. After treatment with petroleum ether for 2 h and redrying, the final weight, corresponding to the muscle fat free dry solids (FFS), was measured. The fragment was extracted with 10 N NaOH for 24 h, then treated according to Cotlove[12] for measurement of the muscle Cl (Cl_mM/kg FFS) with a Buchler Cotlove chloridometer. The partition into extra (ECW) and intracellular (ICW) water was estimated by the chloride method.[7–10,12] Extracellular Cl concentration (Cl_e) was obtained from plasma Cl,[10] and intracellular Cl concentration (Cl_i) was calculated according to the Nernst equation, assuming that resting muscle membrane potential was normal and equal to −87.2 mV.[8–13]

By this procedure, possible abnormalities of membrane potential may introduce only negligible errors in ICW estimation and in the derived values of intracellular AA.[11] ECW was calculated knowing Cl_e, Cl_i, Cl_m, and TW, according to Graham et al.[13]

ICW was given by the difference between TW and ECW; TW, ECW, and ICW were expressed as kg/kg FFS. For each AA, the concentration in ICW (as μmol/kg ICW) was calculated as follows:

$$AA_{ICW} = \frac{AA_m \times (1 + TW) - (ECW \times AA_{ECW})}{ICW}$$

The percent coefficient of variation (CV%) for muscle AA was calculated in five control subjects from whom two independent muscle fragments were taken simultaneously, then processed and analyzed separately. This CV% ($\sqrt{\Sigma[x_1/x_2 - 1]^2/2N} \times 100$, in which x_1 was the greater and x_2 the smaller of the two duplicate values and N the number

TABLE 2
Muscle water, chloride, and intracellular amino acid concentration (μM/kg intracellular water, mean ± SEM) in eight insulin-treated diabetic and nine healthy subjects

	Control subjects (9)	Diabetic subjects (8)
Threonine	623 ± 75	795 ± 71
Serine	1063 ± 165	1767 ± 310
Proline	860 ± 85	2016 ± 158‖
Glycine	1731 ± 196	2253 ± 306
Valine	368 ± 27	633 ± 110*
Alanine	3247 ± 366	3991 ± 299
Methionine	53 ± 4	124 ± 7‖
Isoleucine	103 ± 10	248 ± 60*
Leucine	226 ± 12	285 ± 39
Tyrosine	154 ± 12	187 ± 27
Phenylalanine	120 ± 39	188 ± 27
Ornithine	566 ± 58	622 ± 145
Lysine	1017 ± 97	1196 ± 186
Histidine	553 ± 64	748 ± 66
Arginine	686 ± 86	1019 ± 219
Taurine	18380 ± 1801	16579 ± 1418
BCAA	681 ± 29	1166 ± 219*
Phenylalanine/tyrosine	0.77 ± 0.06	1.12 ± 0.06‡
Intra/extracellular amino acid concentration ratio		
Proline	4.17 ± 0.042	9.65 ± 1.21‖
Valine	1.72 ± 0.09	3.13 ± 0.44§
Isoleucine	1.41 ± 0.12	3.17 ± 0.64†
BCAA	1.69 ± 0.05	2.78 ± 0.36*

Muscle water and chloride	Clm	TW	ICW	ECW
Control (9)	98 ± 7	3.50 ± 0.07	2.77 ± 0.07	0.73 ± 0.07
Diabetic (8)	100 ± 8	3.40 ± 0.06	2.62 ± 0.11	0.77 ± 0.08

Clm = mM/kg FFS, TW, ICW, ECW = kg/kg FFS (muscle fat-free dry solids, mean ± SEM).
*$P < 0.05$, †$P < 0.02$, ‡$P < 0.01$, §$P < 0.005$, and ‖$P < 0.001$.

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of considered cases) took into account not only the variability of analysis, but also that of sampling and processing the muscle fragments;[10] it ranged from 3.2% for valine to 10.4% for methionine. CV% for plasma AA calculated from duplicate analysis in 12 subjects was <4.5% for each measured AA.

The statistical significance was calculated using Student's *t*-test for unpaired data when the population variances were the same. When the variances were different, the analysis was made according to the Wilcoxon test.[14] Equality of the variances was established by the F-test with a 5% level of significance (two-tailed).

RESULTS

The mean blood glucose concentration in the patients examined averaged 1.71 g/L, ketonuria was absent, and daily urinary glucose did not exceed 10 g. Arterial pH and bicarbonate did not differ between diabetic and control subjects. Plasma AA values are summarized in Table 1. Significant differences are found for aspartic acid and ornithine (lower in diabetes) and taurine and phenylalanine/tyrosine ratio (higher in diabetes). Muscle water and chloride contents of muscle from control and diabetic subjects are shown in Table 2. No significant differences were detected between the two groups. Table 2 also reports muscle AA values; mean values for controls were very close to those previously found by other workers.[8]

A significantly higher concentration for proline, methionine, valine, isoleucine, and total BCAA in ICW is evident in the diabetic subjects; the phenylalanine/tyrosine ratio is increased in ICW as is the intra/extracellular ratio for proline, valine, isoleucine, and total BCAA.

DISCUSSION

Plasma AA abnormalities in human diabetes have been extensively investigated.[1,3,4] The concentration of several amino acids has been confirmed to be higher in intracellular than in extracellular fluid;[10] thus, it may be difficult to evaluate the whole body free AA pools in diabetes by only determining their concentrations in plasma water. Muscle tissue is the largest homogeneous cellular mass in the body (45% of the weight in the adult); furthermore, it plays a central role in overall amino acid metabolism and protein turnover.[15] Finally, either uptake and utilization or release of AA by the muscle tissue is well known to be altered in diabetes.[2-5]

Although differences were observed between plasma aspartic acid, taurine, and ornithine concentrations of control and diabetic subjects, most plasma AA concentrations were within the normal range, thus confirming that insulin treatment restores the extracellular AA pattern largely to normal. This is consistent with previous observations that insulin treatment results in a recovery of both plasma low alanine and high BCAA; the pool of intracellular free alanine also seems to be restored by insulin therapy.[6,7] On the other hand, proline, methionine, valine, isoleucine, and total BCAA concentrations in muscle ICW are elevated in insulin-treated diabetic subjects.

Normal extracellular levels and elevated muscle concentrations resulted in increased intra/extracellular gradients for several AA. This was particularly evident for proline and for valine, isoleucine, BCAA as a whole, and methionine, which are specifically transported by the so-called leucine-preferring transport system or system L (BCAA plus methionine, phenylalanine, and tyrosine).[16,17] Indeed, the intra/extracellular ratio of the system L-preferring AA molar sum was 2.93 ± 0.37 in diabetic subjects versus 1.90 ± 0.19 in controls ($P < 0.02$). High plasma and muscle concentrations with elevated muscle/plasma ratio have been previously shown for several system L-preferring AA under different catabolic conditions including starvation, injury, postoperative state, and sepsis.[18-20] The hypothesis has been advanced that these abnormalities are a consequence of an increased overall AA flow from the muscle, due to accelerated net tissue catabolism and to an altered regulation of AA transport.[19,20] It has been reported that in the postabsorptive state, nitrogen balance is more negative in diabetic than in normal subjects.[5] The strong similarities in the muscle/plasma AA distribution between diabetes and catabolic states suggest that this muscle AA pattern in diabetes may be, at least in part, related to the increased muscle protein breakdown.

Although plasma insulin was not measured in this study, low levels of free insulin would be expected under our experimental conditions. On the other hand, in the postabsorptive state the main source of free AA is muscle protein degradation;[15] thus, it is conceivable that high muscle water free AA are related to an accelerated muscle protein catabolism due to insulin deficiency. This mechanism may explain the increase of the free pools of certain AA, but not the elevated intra/extracellular gradients of the system L-preferring AA. It has been recently shown that in the presence of an excess of the system L-preferring, nonmetabolizable AA BCH (2-aminobicyclo-2-2-1-heptane-2-carboxylic acid), the tissue/plasma gradients for valine, isoleucine, leucine, methionine, and proline are increased, rather than decreased, in experimental animals.[21] On this basis the suggestion has been made that system L can mainly promote the exodus of its preferred substrates rather than their entry into the cells.[17,21]

In addition, these AA are actively transported into the cells against their concentration gradient by the Na-dependent, highly concentrative system A.[17,21] Thus, under conditions of increased availability of such free AA, as in diabetes and catabolic states, system A might produce steeper ICW/ECW gradient than system L can sustain. In other words, it may be postulated that the high muscle/plasma ratios reflect a relative saturation of system L in presence of an increased pool of its AA substrates. Our results show a high phenylalanine/tyrosine ratio in both plasma and ICW in diabetic patients, similar to that found under conditions of protein catabolism either acute, as trauma, sepsis,[20] or postoperative state,[19] or chronic, as uremia.[22] It has been previously suggested that a reduced phenylalanine-hydroxylase activity is responsible for this finding. The reduction would be absolute in uremia, as consequence of the lack of this enzyme in failing kidney,[22] or relative, in the presence of an increased flow of phenylalanine from muscle to liver and kidney under acute catabolic events.[19,20] The exact mechanism underlying this decreased rate of conversion of the phenylalanine to tyrosine, however, is not known in diabetes.

In conclusion, our findings show disorders in free AA pools of insulin-treated diabetic patients. These abnormalities are more evident in muscle tissue, where altered AA concentrations may be found in presence of extracellular AA pattern very close to normal. This would further confirm that nor-

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malization of plasma AA is not a satisfactory index of a recovered nitrogen metabolism in diabetes. The striking similarity of this picture to several catabolic conditions supports the hypothesis that the muscle AA abnormalities in insulin-treated diabetes reflect mainly an enhanced protein breakdown due to insulin deficiency.

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