Leucine and Tissue Distribution of Bulky and Small Neutral Amino Acids in Rats: Dissociation between Transport and Insulin-mediated Effects

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Summary: The mechanism of the observed decrease in the plasma concentration of several amino acids in the presence of high levels of Leu has remained unexplained. In the present study a decrease in the plasma concentration of Ile, Val, Phe, Tyr, Met, Ala, Pro and Gly was observed after the intraperitoneal injection of Leu to weanling rats. Decreases in net intracellular concentrations in muscle accompanied the decrease in plasma of all of these amino acids except Pro and Gly. An increase in the distribution ratio muscle/plasma was observed exclusively for Gly after administration of Leu or of a non-insulinogenic transport system L analogue. Diazoxide suppressed the Leu-induced decreases in plasma and muscle intracellular concentrations of Ile and Val as well as of Pro in plasma. An increase in the distribution ratio liver/plasma was observed for Pro and Gly in the absence but not in the presence of diazoxide. All the above changes were statistically significant. Hence insulin probably mediates Leu effects, promoting an increased utilization of Ile and Val in muscle and of Pro in liver. A more direct effect of Leu appears to be involved in the apparent increased utilization of Phe, Tyr and Ala in the same tissue. Gly depletion in plasma can be explained by its trapping by inhibitory action of Leu on the exodus of Gly through transport system L.

High levels of Leu are usually accompanied by a decrease in the plasma concentrations of several amino acids. In maple syrup urine disease (McKusick 24860) (MSUD) Ile and Val are often decreased in plasma in the presence of high Leu levels, in spite of a common metabolic block affecting the catabolism of the three branched-chain amino acids (BCAA) (DiGeorge *et al.*, 1982; Snyderman *et al.*, 1964). Experimental administration of Leu results also in a decrease of Ile and Val, but other, mainly the bulky neutral amino acids also known as large neutral amino acids (LNAA), are also

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depleted in plasma. This effect has been observed in rats fed high-Leu diets (Rogers *et al.*, 1962), after oral loads of Leu to children, either normal or with MSUD (Nyhan *et al.*, 1961; Snyderman *et al.*, 1984), as well as after intravenous Leu infusion to healthy adults (Eriksson *et al.*, 1981) or to patients with hepatic encephalopathy (Rossi-Fanelli *et al.*, 1986). In the latter, the Leu-induced decreases in the plasma concentrations of Phe and Tyr apparently result in clinical improvement (Rossi-Fanelli *et al.*, 1986).

The mechanisms of these decreases have, however, not been explained. Loss in the urine has been excluded as a cause (Nyhan *et al.*, 1961). Indeed, it is likely that different mechanisms may apply to different amino acids or groups of amino acids. Leu is a well-known insulin secretagogue both in humans (Fajans *et al.*, 1967) and rats (Landgraf *et al.*, 1974), and insulin has long been known to cause a decrease in the concentrations of various amino acids in plasma (Luck *et al.*, 1928), the BCAA being the most sensitive (Fukagawa *et al.*, 1986). Transport effects have been proposed but not demonstrated (Nyhan *et al.*, 1961; Eriksson *et al.*, 1981). Eriksson *et al.* (1981) have suggested that exchange diffusion, a distinctive property of transport system L (Oxender and Christensen, 1963; Winter and Christensen, 1964), between high intracellular Leu and plasma LNAA may be involved. Previous work in this laboratory has shown, furthermore, that system L in muscle (Christensen *et al.*, 1948) and liver (Christensen and Cullen, 1981) apparently serves extensively for exodus of several neutral amino acids, previously concentrated into the cell by Na^+-dependent transport systems (Oxender and Christensen, 1963) that operate mainly inwards in these tissues. One of us (H.N.C.) has proposed that this effect may explain the ability of Leu to decrease the neutral amino acids in plasma (Christensen, 1987). In fact, we have shown recently that this inhibitory mechanism applies for various small neutral amino acids, explaining for this group a similar decreasing effect of the Phe on their plasma levels (de Céspedes *et al.*, 1989).

The aim of the present study was to test this hypothesis as well as to assess the role of insulin in the aforementioned effect of Leu on other plasma amino acids.

EXPERIMENTAL PROCEDURES

Materials: L-Leucine was obtained from Sigma Chemical Co. The racemic aminoexo isomer of the 2-aminonorbornane-2-carboxylic acid, aminoexo-2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid, often designated as a($\pm$)BCH, but abbreviated here as aBCH, was our own preparation (Tager and Christensen, 1972). A commercial preparation of diazoxide (Hyperstat IV, Schering Corp.) was used. All other reagents were of the best grade commercially available.

Animals: Twenty two-day-old Sprague–Dawley male weanling rats weighing 38–66 g were used for all the experiments. Rats were fasted for 20–24 h before each experiment.

Procedure: Leu or aBCH was injected intraperitoneally as an isotonic solution at a dose of 10 mmol/kg body weight. Control rats received an intraperitoneal injection

of 0.85% NaCl (w/v) in the corresponding volumen. Diazoxide was also injected intraperitoneally, at a dose of 200 mg/kg body weight 15 min before Leu; control rats received an intraperitoneal injection of distilled water in the corresponding volumen (in no case higher than 0.7 ml).

Two hours after the Leu or aBCH injection the rats were anaesthetized with ether. Blood was taken from the exposed heart and collected in a heparinized tube. Plasma was separated and deproteinized with an equal volumen of 12% sulphosalicylic acid. Approximately 1 g of liver and 0.5 g of muscle from the left thigh were weighed and ground in a mortar containing sand and a 10% solution of sulphosalicylic acid at a volume 4 times the fresh weight of the tissue in grams. The homogenate was filtered through Whatman No. 1 paper. The protein-free filtrate was stored at $-20°C$ until analysis.

Analysis: Amino acids were measured with a model 119 CL Beckman automatic amino acid analyser using a high-sensitivity standard solution from Sigma. Insulin was measured by radioimmunoanalysis, using a species-specific rat antibody, and glucose spectrophotometrically in the Ligand and the Biochemistry Core Laboratories, respectively, of the Diabetes Center of the University of Michigan Medical Center, Ann Arbor.

Calculations: For calculations of apparent concentrations of amino acids in cell water, the liver and muscle samples were taken to contain, respectively, 49% and 62% intracellular and 22% and 15% extracellular water (Christensen and Cullen, 1981; Lowry and Hastings, 1942). The distribution ratio tissue/plasma was the ratio μmol/kg cell water to μmol/kg extracellular water (Lowry and Hastings, 1942).

Statistical analysis: Differences in plasma levels, intracellular concentrations and distribution ratios between rats treated with Leu, Leu and diazoxide, or aBCH, and control rats were calculated by the *t*-test using separate variances when the number of controls and treated animals differed (Wilkinson, 1986).

RESULTS

Plasma levels, net intracellular concentrations and distribution ratios of the amino acids studied after Leu administration, in the absence and in the presence of diazoxide are shown in Table 1. Measurements were made in all cases at 2 h after injection because Ile, an amino acid whose distribution is well known to be particularly sensitive to Leu, was found in preliminary experiments to be strongly decreased in plasma by that time (data not shown).

Plasma levels as well as net intracellular concentrations in both liver and muscle of Leu itself showed statistically significant increases after Leu injection. These increments in Leu levels were about 3–5 times higher in the presence of diazoxide. The distribution ratio muscle/plasma for Leu showed a statistically significant decrease in the absence of diazoxide; otherwise the distribution ratios for Leu appeared unaffected.

Table 1   **Changes in relative concentrations and distribution of each of nine neutral amino acids produced after injecting Leu or Leu plus diazoxide into weaning rats**

	Leu			Leu + diazoxide		
	Plasma	Liver	Muscle	Plasma	Liver	Muscle
Leu	473**	337**	270**	1525*	1621*	1467*
Distribution ratio		0.77	0.58***		1.16	0.97
Ile	36*	101	34*	90	171	82
Distribution ratio		2.68***	0.96		1.74	0.93
Val	34*	118	34**	59	90	51
Distribution ratio		3.99***	1.15		1.67***	0.76
Phe	49**	132	56**	40**	101	43
Distribution ratio		2.83***	1.39		2.64***	1.17
Tyr	43*	119	47**	45*	65	40*
Distribution ratio		2.76**	1.19		1.75	1.06
Met	40*	132	70	47**	–	–
Distribution ratio		3.57**	1.86		–	–
Ala	51*	72	60**	66***	63	70***
Distribution ratio		1.59	1.29		0.96	1.18
Pro	48*	118	70	67	157	73
Distribution ratio		2.51***	1.69		2.14	1.07
Gly	56**	84	89	59**	90	97
Distribution ratio		1.53***	1.61***		1.29	1.53**

The three values in each column show for plasma, liver, and muscle, respectively, the percentage of the control value attained in 2 h for each of these tissues for the amino acid named in column 1. The number below the percentage value for each amino acid shows the calculated relative distribution ratio between the concentrations in the cellular and extracellular water, as described under Methods, for the indicated solid tissue. The number of animals varies from 6 to 7, both for control and experimental groups. Asterisks indicate the statistical significance of changes: $*p < 0.001$, $**p < 0.01$, $***p < 0.05$

Without exception every other amino acid studied showed a statistically significant decrease in its plasma concentration after Leu injection. Only in the case of the other two branched-chain amino acids Ile and Val as well as of Pro was the decrease in plasma concentration prevented by diazoxide. Ile and Val showed as well a statistically significant decrease in their net intracellular concentration in muscle that was prevented also by the previous injection of diazoxide. Distribution ratios of Ile and Val were perturbed in the liver, but not in muscle; the observed statistically significant increase in liver was not prevented by diazoxide.

The aromatic amino acids Phe and Tyr showed also a statistically significant decrease in their net intracellular concentrations in muscle, not prevented by diazoxide. Distribution ratios for Phe, Tyr and Met were also increased in liver but prevented by diazoxide only in the case of Tyr. Tissue Met co-eluted with Cys in the experiments with diazoxide and hence could not be measured.

Ala showed a statistically significant decrease in its net intracellular concentration in muscle, an effect insensitive to diazoxide. Pro and Gly showed a statistically significant increase in their respective distribution ratios for liver that was preventd by diazoxide. Gly and Pro were, among all other amino acids studied (Leu of course excluded from consideration), the only ones that did not show a statistically significant

decrease in their net intracellular concentration in muscle. Gly, on the other hand, was the only amino acid that showed a statistically significant increase in its distribution ratio in muscle after Leu administration. This effect was insensitive to diazoxide.

Experiments parallel to those with Leu were made with the system L analogue aBCH (Figure 1). This isomer of BCH was selected because it is not an insulin secretagogue (Tager and Christensen, 1971) and hence could serve to dissociate that action from the suspected direct effects on amino acid transport (Oxender and Christensen, 1963; Christensen, 1987). Gly was the only amino acid that showed a statistically significant decrease in plasma concentration after aBCH. The only statistically significant change found in liver after aBCH was a decrease in the net intracellular concentration of Gly (data not shown). On the other hand, aBCH caused in muscle a statistically significant increase in the distribution ratios of Ala and Gly, reproducing the same distinctive effect of Leu for the latter (Figure 1).

The effect of Leu in the absence and in the presence of diazoxide on plasma glucose and insulin concentrations at different times is shown in Figure 2. As expected, Leu caused a decrease in the plasma concentration of glucose that was prevented by diazoxide (Figure 2A). The insulin/glucose ratio was increased by 345% of the control at 60 min after Leu injection; this effect was also largely prevented by diazoxide (Figure 2B).
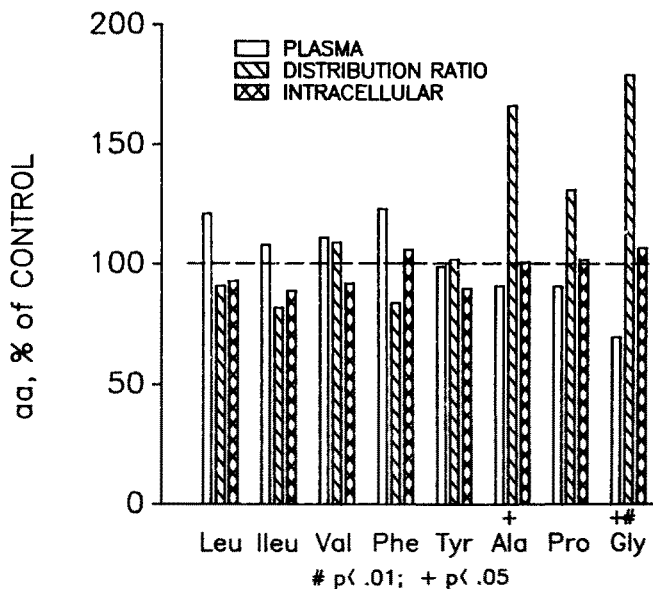

Figure 1 Plasma levels, distribution ratios, tissue/plasma, and net intracellular concentrations for muscle of neutral amino acids after aBCH injection. Data are shown as percentage of controls. N varied from 7 to 13 animals for both controls and experimental groups
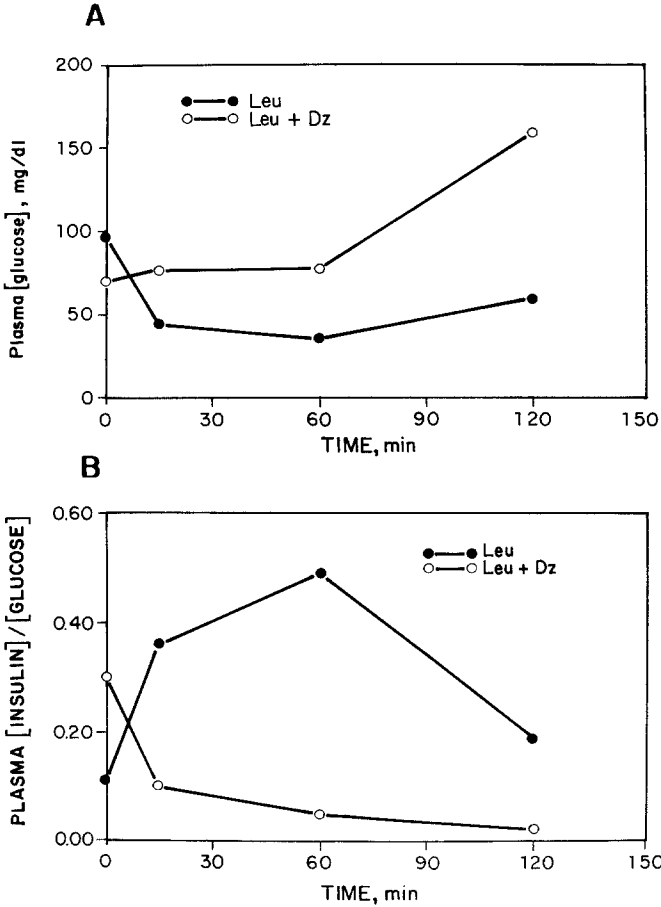
A

B

Figure 2 Time course of glucose concentration (A) and of insulin/glucose ratio (B) in plasma after Leu injection in the absence or in the presence of diazoxide (Dz). Each point represents the average for three animals

DISCUSSION

Conceivably, Leu may cause a decrease in the plasma concentration of other amino acids by promoting their utilization in tissues directly (Buse and Weigand, 1977) or through insulin (Fulks *et al.*, 1975). Membrane transport effects have also been proposed (Eriksson *et al.*, 1981; Nyhan *et al.*, 1961) although they have received little attention. Insulin could also act by stimulating membrane transport (Shotwell *et al.*, 1983).

Under our experimental conditions, the amino acids studied were likely to be exposed to an insulin effect, according to the time course of glucose and insulin concentration changes. Although the latter were modest (data not shown), it is accepted that insulin variations in plasma are more properly interpreted by measuring the glucose levels simultaneously (Young and Kavam, 1991). Accordingly, the sharp increase in the insulin/glucose ratio and its prevention by diazoxide points to an

insulin-releasing effect of Leu (Figure 2B). This is consistent with previous work that showed that Leu is a potent insulin secretagogue in the rat (Landgraf *et al.*, 1974). Although a direct effect cannot be ruled out at this time, it is likely that the effects of diazoxide observed here are due to its well known inhibitory effect on insulin release (Wolff *et al.*, 1968).

The selective sensitivity of some of these amino acids to diazoxide is, in fact, consistent with insulin effects in humans. Fukagawa *et al.* (1986), using the euglycaemic insulin clamp technique, showed that Ile and Pro were the most sensitive among several amino acids. Moreover, in the same work these authors found that Gly and Ala concentrations in plasma were unaffected by insulin, as was observed in the present study. Contrary to our findings, however, they found a statistically significant decrease in Phe and Tyr plasma concentrations after insulin infusion (Fukagawa *et al.*, 1986). We believe that a possible insulin contribution to the decrease in the plasma concentrations of Phe and Tyr in the present study was masked by a stronger effect of Leu in the utilization of these two aromatic amino acids. This interpretation is consistent with the observed enhanced incorporation of [^{14}C]Tyr into protein, promoted by Leu in isolated rat diaphragm (Buse and Weigand, 1977). The decrease of Ala in plasma, clearly insulin-independent in this study, can also be explained by a direct effect of Leu on the utilization of this amino acid in muscle, as could be the case for Met also.

Eriksson *et al.* (1981) found a small but statistically significant increase and decrease in plasma insulin and glucose concentrations, respectively, after Leu infusion to healthy human adults. Concomitantly these authors found a statistically significant decrease in the plasma levels of all the amino acids studied here, among others. However, they raised the possibility of stimulated exchange diffusion of the intracellular Leu with the plasma large neutral amino acids through transport system L (Eriksson *et al.*, 1981). Since we were particularly interested in net changes in amino acid distribution, our experimental design here does not allow us to exclude such a mechanism of stimulated exchange diffusion, which may be a very early event after Leu administration. In the present study, Gly was the only amino acid that showed a statistically significant increase in its distribution ratio muscle/plasma (Table 1). The parallel finding that only Gly showed a statistically significant decrease and increase, respectively, in its plasma concentration and in its distribution ratio muscle/plasma after aBCH administration (Figure 1) strongly suggests that the corresponding decrease of Gly in plasma caused by Leu is due to trapping of this amino acid by inhibition of its exodus from muscle cells through system L, according to the hypothesis proposed here. This explanation is again consistent with early and recent work in our laboratory showing that Gly, Ala and Pro seem particularly prone to be trapped into muscle and liver cells in the presence of excesses of Phe (de Céspedes *et al.*, 1989, their figs. 3 and 4) and other system L substrates or analogues (Christensen *et al.*, 1948). This effect probably arises at least in part from the readiness of the entry of the small neutral amino acids through Na$^+$-dependent system A, a movement parallel, but in the opposite direction, to the one occurring through system L in muscle and liver (Christensen, 1987; de Céspedes *et al.*, 1989). Accordingly, the statistically significant decrease in Pro plasma concentration may be explained by

transport effects as shown by the statistically significant increase in its distribution ratio liver/plasma. Since the distribution ratio liver/plasma for Gly also showed a statistically significant increase, its trapping in liver may make an additional contribution to its decrease in plasma. Moreover, this apparent enhancement of transport of Pro and Gly in liver is not observed in the presence of diazoxide. Since transport system A has been shown to be responsive to insulin in isolated preparations of liver and muscle (Shotwell *et al.*, 1983), a role for insulin in stimulating the entry of Pro and Gly through system A in liver seems likely. Although not statistically significant, the increase in the distribution ratio liver/plasma, and its relative decrease in the presence of diazoxide, is also observed for Ala.

We have no explanation at this time for the mechanism of the increased distribution ratios liver/plasma for Ile, Val, Phe, Tyr and Met that otherwise is probably contributing to the depletion of these amino acids in plasma. We do not know either why these increases, with the exception of Tyr, are all unaffected by diazoxide.

A stimulatory effect of the enzymes of the branched-chain amino acid catabolic pathways has been proposed as a mechanism of the Leu-induced decrease in the plasma concentrations of Ile and Val (Phansalker *et al.*, 1970). If this mechanism were to operate in MSUD, some residual stimulative activity of the branched-chain ketoacid decarboxylase should still be present. Insulin levels have been reported as normal in patients with MSUD (Soltesz *et al.*, 1983). Since in the present study, the observed Leu-induced decrease of Ile and Val in plasma and muscle was prevented by diazoxide, insulin seems to play a role in this effect, at least under our experimental conditions. Utilization of Leu itself in liver and muscle, probably for protein synthesis (Tessari *et al.*, 1987), is apparently enhanced by insulin, since much higher levels were observed in those tissues as well as in plasma in the presence of diazoxide.

Distinguishing among the above mechanisms may be important in guiding attempts to correct amino acid imbalances in hereditary or acquired disease states that may lead to short- or long-term deleterious effects, especially on the brain. We have discussed this subject (Christensen, 1987; de Céspedes *et al.*, 1989). A compensation for such effects might be attained by the administration of selected amino acids or amino acid analogues, or of insulin or insulin antagonists. Further work is needed, however, to assess the significance of the depletion of plasma neutral amino acids, some of them (such as Phe and Tyr) precursors of neurotransmitters or themselves neurotransmitters as is Gly, as a cause of impairment of the function or the structure of the brain.

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SHORT REPORT

Alloisoleucine in isovaleric acidaemia

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L-Alloisoleucine (L-aIle) is the isomer of the protein amino acid L-isoleucine (L-Ile). This non-protein amino acid has been reported in high concentrations in the plasma of patients with branched-chain ketoacid (BCKA) dehydrogenase deficiency (maple syrup urine disease) or of patients receiving a diet supplemented with BCKA. In this paper we report the presence of L-aIle in the plasma of three patients with isovaleric acidaemia (IVA) (McKusick 24350).

These patients, D.A., R.P. and R.A., were 27, 13 and 13 months old respectively; R.P. and R.A. are twins. They were diagnosed during the first week of life by gas chromatography–mass spectrometry. These children received a protein-restricted diet during the first 6 months of life and then a normal diet. This diet was supplemented by a mixture of amino acids lacking L-Leu (from 15 days to 18 months for D.A. and between 15 and 20 days of age for R.P. and R.A.). All the patients received glycine ($250 \, mg \, kg^{-1} \, day^{-1}$) and L-carnitine ($100–200 \, mg \, kg^{-1} \, day^{-1}$). Their plasma amino acids are regularly controlled by ion-exchange chromatography (Beckman 6300 System). The mixture of amino acids used as nitrogen supplementation was checked for the absence of L-Leu and L-aIle. For these three patients L-aIle was undetectable during the first 2 weeks of life. After this time L-aIle ranged from 2 to $10 \, \mu mol/L$ (controls undetectable). When L-aIle was undetectable, L-Ile concentration was very low (range $18–28 \, \mu mol/L$; control mean $\pm$ SD 64 ± 15 and range $35–101$, $n = 174$).

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