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Amino Acids

Blood amino acids concentration during insulin induced hypoglycemia in rats: the role of alanine and glutamine in glucose recovery

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Summary. Our purpose was to determine the blood amino acid concentration during insulin induced hypoglycemia (IIH) and examine if the administration of alanine or glutamine could help glycemia recovery in fasted rats. IIH was obtained by an intraperitoneal injection of regular insulin (1.0 U/kg). The blood levels of the majority of amino acids, including alanine and glutamine were decreased ($P < 0.05$) during IIH and this change correlates well with the duration than the intensity of hypoglycemia. On the other hand, the oral and intraperitoneal administration of alanine (100 mg/kg) or glutamine (100 mg/kg) accelerates glucose recovery. This effect was partly at least consequence of the increased capacity of the livers from IIH group to produce glucose from alanine and glutamine. It was concluded that the blood amino acids availability during IIH, particularly alanine and glutamine, play a pivotal role in recovery from hypoglycemia.

Keywords: Amino acids – Alanine – Glutamine – Gluconeogenesis – Hypoglycemia – Insulin

Introduction

Intensified insulin therapy in type 1 (DCCT, 1993) and type 2 diabetes (UKPDS, 1998) has been show to improve metabolic control and prevent chronic complications. However, the DCCT and UKPDS studies also shown a relationship between tight blood glucose control and the development of insulin induced hypoglycemia (IIH). These studies suggest that strategies to achieve normal or near normal glycemia, which protect the patient against chronic complications, are limited by an increased number of acute episodes of IIH (Davis and Alonso, 2004).

To understand better the mechanism of IIH we developed a rat model in which hypoglycemia was produced by an intraperitoneal administration of regular insulin in fed (Lopes et al., 1998; Vardanega-Peicher et al., 2003) and fasted rats (Souza et al., 2001a, b). In addition, we observed that an increase in blood free fatty acids was associated with glucose recovery (Souza et al., 1996).

On the other hand in spite of the abundant research on amino acids metabolism, the data provide little information on changes of amino acids concentration in the blood during IIH (Nair et al., 1990; De Feo et al., 1992; Godil et al., 2005). Thus, the purpose of this study was to investigate the effect of short-term and long-term IIH on the blood concentration of amino acids in fasted rats. In addition, the acute effect of the administration of the most important gluconeogenic amino acid, i.e., alanine (Pilks and Granner, 1992) and the most abundant extracelullar amino acid, i.e., glutamine (Newsholme et al., 2003) on glycemia recovery was investigated.

Materials and methods

Animals

Male Wistar 24-h fasted rats (*Rattus norvegicus*) weighing 180–220 g were used in accordance with the Brazilian law on the protection of animals. The rats were maintained at constant temperature (23 °C) with automatically controlled photoperiod (12-h light/12-h dark). To minimize circadian variations all experiments started at 8:00 a.m.

Materials

Regular insulin (Novolin® R) was obtained from Novo-Nordisk, Brazil. Alanine and glutamine were obtained from ICN, USA. All other reagents were of the highest purity obtainable.

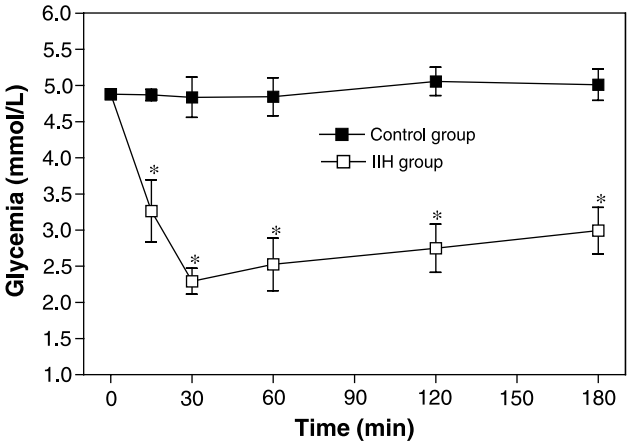

Fig. 1. Glycemia 0, 15, 30, 60, 120 and 180 min after intraperitoneal injection of insulin (IIH group, □) or vehicle (Control group, ■) of 24-h fasted rats. Data were expressed as means ± SD of 6–8 animals. $^{*}P < 0.05$ vs. 0 min

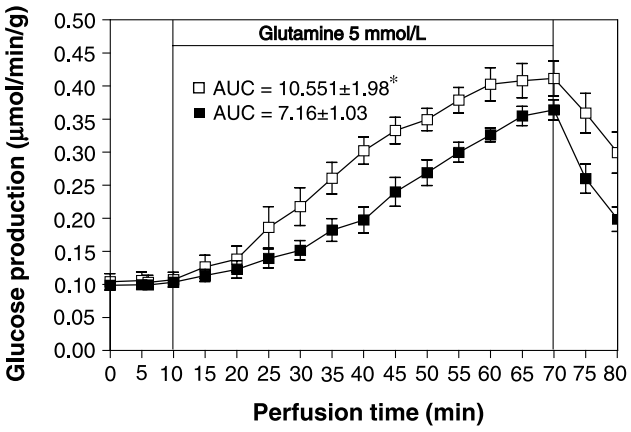

Fig. 2. Demonstrative experiment of glucose production from glutamine in perfused livers from 24-h fasted rats that received insulin (IIH group, □) or vehicle (Control group, ■). The effluent perfusate was collected in 5 min intervals and analysed for glucose. Data expressed as means ± SD of 6–8 experiments. *AUC* Area under the curves (µmol/g). $^{*}P < 0.05$ vs. Control group

Experimental IIH

The experiment to characterize IIH after an intraperitoneal injection of regular insulin (1.0 U/kg) is illustrated by Fig. 1. The rats were killed by decapitation 0, 30, 60, 120 and 180 min after insulin (IIH group) or saline (Control group) administration. Blood was collected and centrifuged for 15 min (3000 rpm) to separate cells from serum, which was frozen until time of analysis. Serum (10 µl) was used for glucose determination (Bergmeyer and Bernt, 1974). The data shown that glycemia progressively decreased from a baseline value until 30 min and then progressively increased until 180 min after insulin administration (IIH group). In contrast, the values after vehicle injection (control group) were unchanged until 180 min. In addition, considering that hypoglycemia was well-established by 30 min after insulin administration and was maintained until 180 min, both times were selected for measurement of blood levels of amino acids. The basal blood concentration of glutamine and alanine obtained from IIH and control groups (Table 1) were used in the liver perfusion experiments.

Amino acid profile

For amino acid analysis, serum samples (1 ml) were deproteinized with 25% trichloroacetic acid (TCA; 1 ml). After centrifugation (10,000 × *g*), 30 µl of the supernatant was analyzed on a Biochem 20 plus amino acid analyzer (Amersham Pharmacia, Piscataway, NJ), using a PEEK cation-exchange high-performance column with ninhydrin detection. Amino acids were quantified, using an amino acid standard (Sigma), as previously described (Delghingaro-Augusto et al., 2004) by Biochrom 20 control software version 3.05.

Oral and intraperitoneal administration of amino acids on glucose recovery

Glutamine (100 mg/kg) or alanine (100 mg/kg) were orally (gavage) or intraperitoneally administered 165 min after insulin (IIH group) injection. Moreover, an additional group that received oral or intraperitoneal vehicle was included. Blood samples were collected by decapitation 15 min later, i.e., 180 min after insulin injection and glycemia was measured by the glucose oxidase method (Bergmeyer and Bernt, 1974).

Liver perfusion technique

The rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and submitted to laparotomy. The livers were perfused as previously described (Murad et al., 2004) according to the protocol illustrated in Fig. 2. After a pre-perfusion period (10 min), glutamine was dissolved in the perfusion fluid and infused between the 10th and 70th min of the perfusion period, followed by a period of post-infusion (10 min) to allow the return to basal glucose production. Samples of the effluent perfusion fluid were collected at 5 min intervals, and glucose production was determined (Bergmeyer and Bernt, 1974). The differences in the glucose production during (10–70 min) and before (0–10 min) the infusion of glutamine allowed calculating the areas under the curves (AUC), expressed as µmol/g. A similar procedure was performed when alanine was used as the gluconeogenic substrate. Moreover, in part of the liver perfusion experiments lactate (Gutmann and Wahlefeld, 1974), pyruvate (Czok and Lamprecht, 1974) and urea (Gutmann and Bergmeyer, 1974) production were measured. Thus, the AUCs shown in Fig. 4 were obtained from experiments similar to that shown in Fig. 2.

Determination of the liver capacity to produce glucose from glutamine or alanine

Experiments using increasing levels of glutamine or alanine were performed. The addition of the amino acids increased the rate of glucose production in a dose-dependent manner, proportional to the amount of precursor, until the liver capacity was reached, i.e., the lowest concentration at which the maximal hepatic glucose production was obtained. The value obtained for alanine and glutamine, i.e., 5.0 mmol/l (result not shown) was used in the liver perfusion experiments (Fig. 4).

Statistical analysis

Results are reported as means ± SD. The program GraphPad Prism (version 3.0) was used to calculate the AUC. Data were analyzed statistically by an unpaired Student's *t*-test. $P < 0.05$ were accepted for all comparisons.

Results

The levels of the following amino acids and related compounds were determined: alpha-aminoadipic acid, alpha-aminobutiric acid, alpha-phosphoethanolamine,

Table 1. Blood concentration (mmol/l) of amino acids 30 (**A**) and 180 min (**B**) after intraperitoneal injection of vehicle (Control group) or insulin (IIH group). Data were expressed as means ± SD. $^{*}P<0.05$ vs. control group

	Control group	IIH group	% of change
A (30 min)			
Cysteine	0.05 ± 0.01	0.00 ± 0.0*	100
Glutamate acid	0.33 ± 0.04	0.24 ± 0.03*	27
Glutamine	2.00 ± 0.21	1.41 ± 0.10*	30
Glycine	0.86 ± 0.02	0.58 ± 0.03*	32
Isoleucine	0.15 ± 0.01	0.11 ± 0.02*	27
Methionine	0.34 ± 0.02	0.07 ± 0.02*	79
Phenylalanine	0.12 ± 0.01	0.07 ± 0.00*	42
Proline	0.16 ± 0.01	0.12 ± 0.02*	25
Valine	0.40 ± 0.03	0.30 ± 0.04*	25
B (180 min)			
Alanine	0.45 ± 0.07	0.22 ± 0.02*	51
Asparagine	0.09 ± 0.02	0.04 ± 0.02*	55
Citrulline	0.10 ± 0.01	0.06 ± 0.07*	40
Glutamate acid	0.20 ± 0.02	0.14 ± 0.02*	30
Glutamine	1.29 ± 0.04	0.70 ± 0.12*	46
Glycine	0.54 ± 0.05	0.32 ± 0.04*	41
Isoleucine	0.11 ± 0.01	0.06 ± 0.01*	45
Leucine	0.24 ± 0.01	0.17 ± 0.03*	29
Lysine	0.45 ± 0.07	0.31 ± 0.05*	31
Proline	0.06 ± 0.02	0.02 ± 0.00*	67
Serine	0.26 ± 0.03	0.17 ± 0.03*	35
Threonine	0.29 ± 0.04	0.18 ± 0.02*	38
Tyrosine	0.11 ± 0.01	0.06 ± 0.01*	45
Valine	0.25 ± 0.03	0.20 ± 0.04*	20

alpha-phosphoserine, alanine, arginine, asparagine, aspartate, citrulline, cysteine, glutamate acid, glutamine, glycine, hydroxylysine, histidine, isoleucine, leucine, lysine, methionine, 1-methyl-histidine, 3-methyl-histidine, ornitine, phenylalanine, proline, sarcosine, serine, taurine, threonine, tyrosine and valine. However, only those compounds that showed significant ($P<0.05$) decrease during IIH are included in Table 1A and B. The blood concentration of glutamine (but not alanine), cysteine, glutamate acid, glycine, isoleucine, methionine, phenylalanine, proline and valine were decreased ($P<0.05$) 30 min after insulin administration (Table 1A). In addition we observed that the blood concentration of alanine, asparagine, citruline, glutamate acid, glutamine, glycine, isoleucine, leucine, lysine, proline, serine, threonine, tyrosine and valine were decreased 180 min after insulin injection (Table 1B).

A better glucose recovery was observed if alanine or glutamine were offered (orally or intraperitoneally) 165 min after insulin injection (Fig. 3A and B). In agreement with these results the hepatic production of glucose (Fig. 4A), urea (Fig. 4B), lactate (Fig. 4C) and pyruvate (Fig. 4D) were markedly increased ($P<0.05$) when the livers were perfused with saturating levels of alanine or glutamine if compared with the basal concentration (Table 1B) of these amino acids. In addition, livers from IIH group showed higher ($P<0.05$) capacity to produce glucose, urea, pyruvate and lactate from saturating concentration of alanine or glutamine if compared with livers of control group.

Discussion

Because the decreased blood levels of amino acids correlates better with the duration than the intensity of IIH (Table 1A and B) we investigate the participation of the blood amino acid availability to the liver in the glycemia recovery. For this purpose, alanine and glutamine were

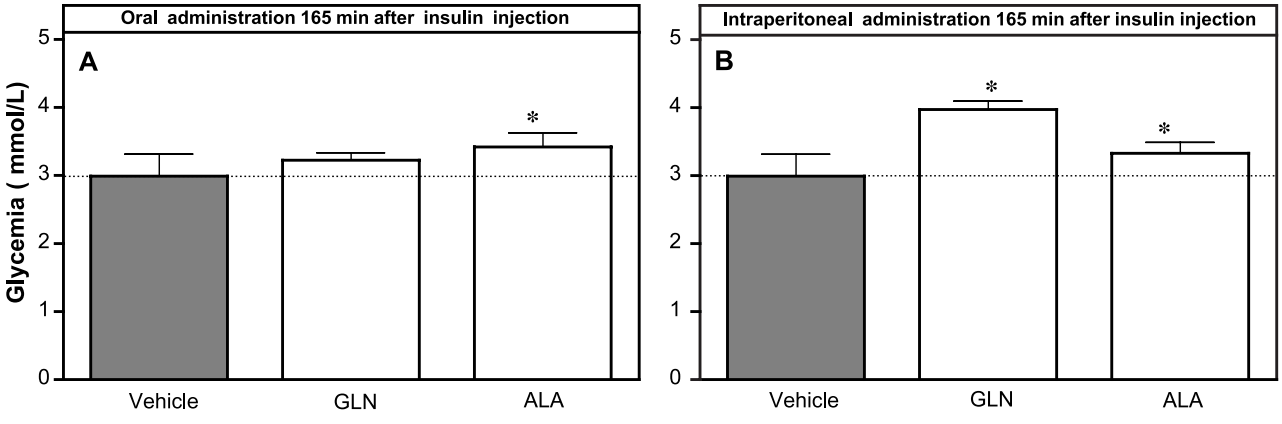

Fig. 3. Effect of oral (**A**) and intraperitoneal (**B**) administration of vehicle, 100 mg/kg glutamine (GLN) or 100 mg/kg alanine (ALA) on glycemia of 24-h fasted rats. The amino acids were administered 165 min after insulin injection. Glycemia was measured 15 min later, i.e., 180 min after insulin injection. Data were expressed as means ± SD of 6–8 experiments. $^{*}P<0.05$ vs. vehicle-IIH group

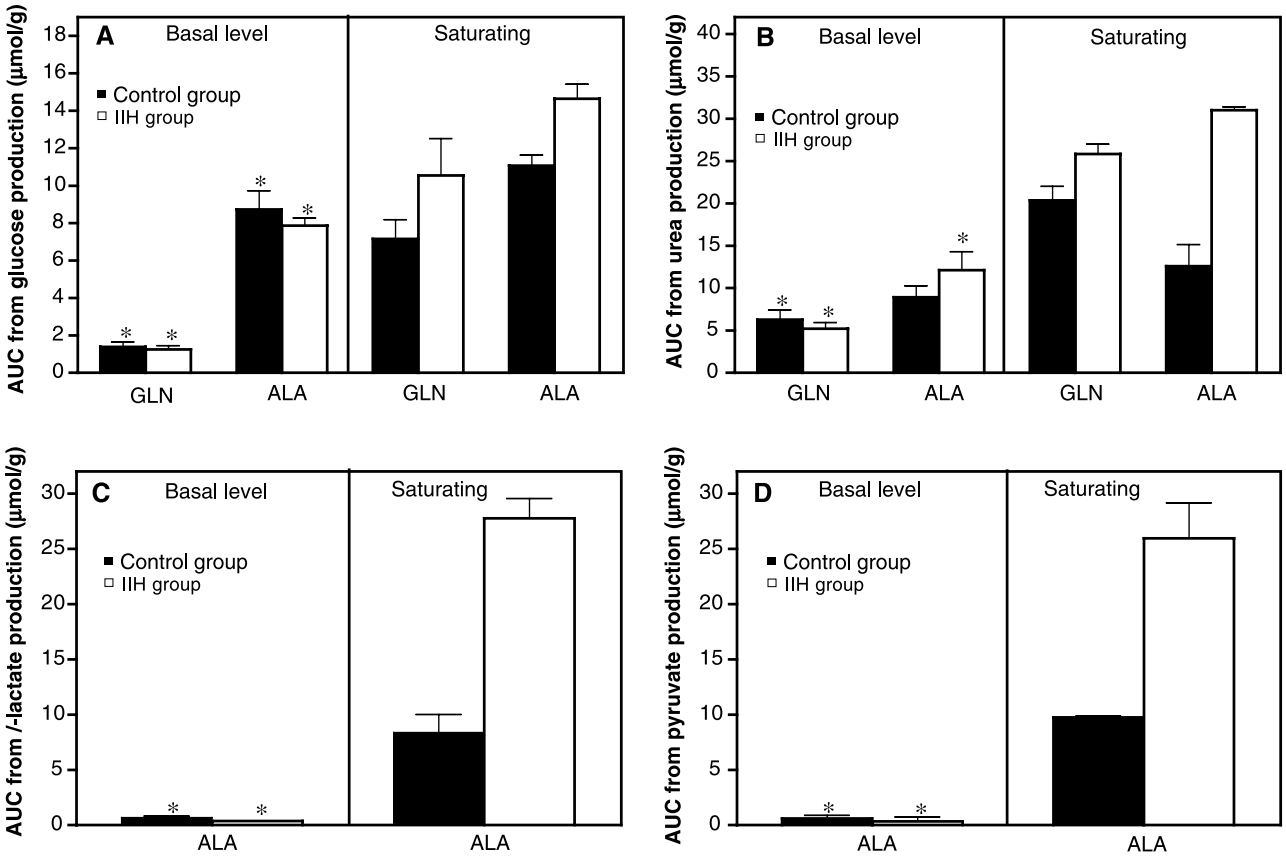

Fig. 4. Effect of basal and saturating concentration of alanine (ALA) or glutamine (GLN) on: **A** glucose; **B** urea; **C** lactate, **D** pyruvate production in livers from hypoglycemic (IIH group, □) and normoglycemic (Control group, ■) 24-h fasted rats. The livers were perfused as described in Materials and methods. Data were expressed as means ± SD of 6–8 experiments. *AUC* Area under the curves. $^{*}P < 0.05$ vs. saturating concentration

orally and intraperitoneally administered 165 min after insulin injection, and glycemia was measured 15 min later, i.e., 180 min after insulin injection. As shown in Fig. 3 both amino acids promoted glucose recovery. The worst result, obtained with oral glutamine could be ascribed to the fact that the intestinal cells utilize this amino acid at high rates (Newsholme et al., 2003) reducing the amount disposable to the liver. In agreement, the intraperitoneal administration of glutamine (Fig. 3B) which overcomes the influence of intestinal metabolism showed better results than oral glutamine (Fig. 3A). In contrast, the best glycemia recovery obtained with oral alanine could be attributed not only to its increased portal availability (Newsholme et al., 2003) but also an intensification of glucagon secretion (Wiethop and Cryer, 1993).

In contrast with data obtained on administration of alanine and/or glutamine (Fig. 3), our previous studies (Souza et al., 2001a, b) showed that the administration of glucose during IIH did not promote glucose recovery. In addition, we demonstrated that this effect was partly at least due to the inhibition of hepatic gluconeogenesis. Thus, these results have clinical significance considering that glucose administration is commonly used for the management of hypoglycemia (Moore and Woollard, 2005).

Hepatic glucose production increased ($P < 0.05$) several fold when the livers were perfused with saturating concentration of glutamine or alanine (Fig. 4A). These results were the consequence of increased catabolism of both amino acids as inferred by the higher ($P < 0.05$) urea (Fig. 4B), lactate (Fig. 4C) and pyruvate (Fig. 4D) production. In addition, higher values ($P < 0.05$) of glucose production from saturating concentration of alanine and glutamine were observed in livers from IIH group (Fig. 4). Thus, the liver's ability to produce glucose from a saturating level of alanine or glutamine was increased in livers from IIH group. In agreement with this suggestion, livers from IIH group showed a favorable redox potential for gluconeogenesis, i.e., an increased NADH/NAD cytosolic ratio, inferred by the higher ($P < 0.05$) lactate (Fig. 4C) and lower ($P < 0.05$) pyruvate (Fig. 4D) production. Similar results were obtained when the experiments were performed 30 min after insulin administration (not shown). The mechanism

involved in the increased capacity in producing glucose during IIH also involves the increased release of counter-regulatory hormones (Cryer and Polonsky, 1998) which stimulate the uptake and catabolism of amino acids and activates the enzymes alanine aminotransferase, glutaminase, phosphoenol-pyruvate carboxykinase and glucose-6-phosphatase (Lea et al., 2001; Cynober, 2002; Jiang and Zang, 2002; Nissim et al., 2003).

Overall, the increased hepatic capacity to produce glucose from alanine and glutamine during IIH suggests that oral alanine or parenteral glutamine could help glucose recovery. Another interesting possibility, is the use of both amino acids to prevent (M'bemba et al., 2003) IIH. However, the prospect of using these amino acids to prevent IIH await further experimental and clinical studies.

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