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Oral Glutamine Dipeptide Promotes Acute Glycemia Recovery in Rats Submitted to Long-Term Insulin Induced Hypoglycemia

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SUMMARY. The acute effect of oral administration (100 mg/kg) of glutamine dipeptide (L-alanyl-L-glutamine, AGP), L-alanine (ALA), or L-glutamine (GLN) on glycemia recovery (GR) during long-term insulin induced hypoglycemia (IIH) was compared. Thus, 24-h fasted rats that received intraperitoneal regular insulin (1.0 U/kg) and 165 min later oral AGP, ALA, GLN or saline (control group) were compared. Glycemia was measured 180 min after insulin administration. In contrast with GLN, oral AGP or ALA promoted GR. However, the participation of the liver metabolism to GR, particularly the augmented hepatic glucose production was less intense to the group that received oral AGP (AGP *vs.* ALA). We concluded that the oral AGP promoted acute glycemia recovery with less intense participation of liver gluconeogenesis.

RESUMEN. "El Dipéptido de Glutamina administrado por Vía Oral promueve la Recuperación de Glucemia Aguda en Ratas Sometidas a Hipoglucemia por Insulina durante largo tiempo". Fue comparado el efecto agudo de la administración oral (100 mg/kg) del dipéptido de glutamina (L-alanil-L-glutamina, AGP), L-alanina (ALA) y L-glutamina (GLN) en la recuperación de la glucemia (RG) en los casos de hipoglucemia inducida por insulina (IIH) durante largo tiempo. Se comparó el comportamiento de ratas en ayuno de 24 h que recibieron insulina regular por vía intraperitoneal (1,0 U/kg) y luego de 165 min AGP, ALA, GLN o solución salina por vía oral. La glucemia fue evaluada a los 180 min después de la adminsitración de insulina. A diferencia de GLN, la administración oral de AGP o ALA promovió la RG. Sin embargo, la participación del metabolismo hepático en la RG, particularmente el aumento de la producción hepática de glucosa, fue menos intensa en el grupo que recibió AGP (AGP *vs*. ALA) por vía oral. Se concluye que la administración de AGP por vía oral promovió la recuperación aguad de la glucemia con una participación menos intensa de la gluconeogénesis hepática.

INTRODUCTION

Intensive insulin therapy which prevents the development of the chronic complications of type 1 and type 2 diabetes is associated with an increased incidence of insulin induced hypoglycemia (IIH) which is the major limitation for the implementation of the treatment [1]. Since glucose shows evanescent effect on glucose recovery [2] much effort is being devoted to develop other compounds that could get better results to treat IIH. Among others, we previously showed that the parenteral administration of L-glutamine (GLN) during IIH were more efficient than glucose itself to promote glucose recovery [3]. In contrast, oral GLN did not promote glucose recovery [4] and the reason is the fact that oral GLN suffer intense catabolism by the enterocytes [5] and its liver availability is negligible.

In contrast with GLN, L-alanyl-L-glutamine peptide (AGP), also known by the name of glutamine dipeptide could overcome the catabolism of enterocytes [6,7]. Moreover, considering that AGP, commonly used in parenteral nutrition [8] is composed by the most important gluconeogenic amino acid, *i.e.,* L-alanine (ALA) and the most abundant blood amino acid, *i.e.,* GLN [9], the possibility to obtain glucose recovery by using oral AGP must be considered. In agreement with this proposition intravenous AGP promoted glucose recovery in patients with type 1 diabetes submitted to IIH [10]. However, to the authors' knowledge the acute effect of oral

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AGP on glucose recovery during long- term IIH was never investigated before.

Thus, the objective of the present study was investigate the acute effect of oral AGP on glucose recovery during long-term IIH. Since AGP is made of GLN and ALA, both amino acids were included for comparative purpose. Moreover, the contribution of hepatic gluconeogenesis to glucose recovery was investigated.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats, weighing between 180 and 220 g were maintained on standard rodent chow and water *ad libitum* before the initiation of experimental procedures. The rats were maintained at constant temperature (23 °C) with automatically controlled photo-period (12-h light / 12-h dark). On the day before the experiment, animals were food deprived. All experiments were performed with 24-h fasting rats a favourable condition for gluconeogenesis, at which hepatic glycogen was completely depleted [11].

The experimental protocol was approved by the institutional animal welfare committee (protocol number 008/2006 and approval number 042/2006).

Materials

Regular insulin (Novolin®) and AGP was purchased respectively from Novo Nordisk and Fresenius Kabi Brazil Ltda. ALA and GLN were obtained from ICN. All other reagents were of the highest purity obtainable.

Experimental long-term IIH

A preliminary experiment to characterize the long-term IIH after an ip injection of Regular insulin (1.0 U/kg) was done. The values of glycemia after insulin injection at 0 min, 15 min, 30 min, 60 min, 120 min and 180 min were 91.3 mg/dl, 59.3 mg/dl, 41.7 mg/dl, 45.9 mg/dl, 50.0 mg/dl and 54.4 mg/dl, respectively. Since hypoglycemia was maintained until 180 min after insulin injection, this time was selected to evaluate the acute effect of gluconeogenic amino acids on glucose recovery.

Evaluation of AGP, ALA or GLN administration on glucose recovery

The amino acids AGP (100 mg/Kg), ALA (100 mg/Kg) or GLN (100 mg/Kg) were orally administered (gavage) 165 min after insulin injection. The control group received oral saline 165 min after insulin administration. Blood samples were collected by decapitation and glycemia [12] was measured 15 min later, *i.e.,* 180 min after insulin injection.

Liver perfusion technique

The rats were anaesthetized by an ip injection of thiopental (40 mg/kg) 180 min after insulin (IIH group) or saline (normoglycemic group) injection and submitted to laparotomy. The livers were perfused *in situ* using Krebs-Henseleit bicarbonate buffer (pH 7.4) and saturated with a mixture of O_2/CO_2 (95%/5%). Perfusion fluid was pumped through a temperature-controlled (37 °C) membrane oxygenator prior entering into the liver through the portal vein. Perfusion was performed in an open system with no recirculation of the perfusate [13]. The viability of the liver during the perfusion was indicated by the absence of any leaking and/or tissue swelling. Since liver weight from fasted rats represents about 4% of the body weight, the liver weight was inferred from the rat weight (0.04 x rat weight). Afterwards the flow rate in each experiment was adjusted according to the inferred liver weight (4 ml/g of inferred liver fresh weight/min). After finishing the perfusion experiments the livers were weighted and the metabolite production (glucose, urea, pyruvate and L-lactate) were calculated by using the formula: Metabolite concentration in the effluent (μmol x ml^{-1}) /flux (ml/ ml^{-1}) x liver weight (g).

Evaluation of the maximal liver capacity to produce glucose from AGP, ALA or GLN

Perfused livers from fasted rats produce insignificant amount of glucose in the absence of gluconeogenic precursors. The addition of AGP, ALA or GLN increased the rate of glucose production proportionately to the amount of the glucose precursor until a saturating concentration is reached and it is possible to measure the maximal capacity of the liver to produce glucose from these glucose precursors. The concentration obtained to AGP, ALA or GLN was 5.0 mM (data not shown). This value was used in the liver perfusion experiments according to the protocol illustrated by Fig. 1. As shown in Fig. 1 after a pre-perfusion period (10 min), the gluconeogenic substrate was dissolved in the perfusion fluid and infused during 60 min, followed by a post-infusion period (10 min) to allow the return to basal levels.

Samples of the effluent perfusion fluid were

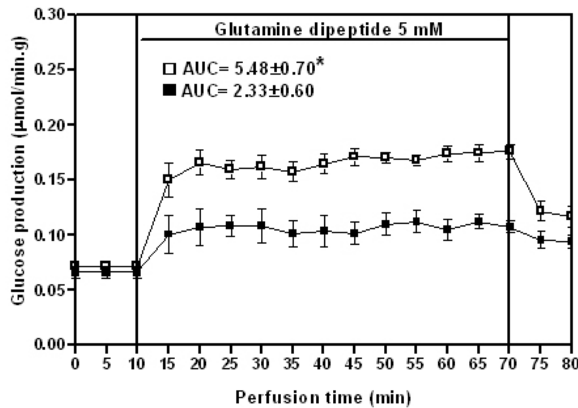

Figure 1. Demonstrative experiment of glucose production from a saturating concentration of glutamine dipeptide (AGP) in livers of 24-h fasted rats that received intraperitoneal injection of insulin (IIH group, ❑ or saline (Normoglycemic group, ■). The effluent perfusate was sampled in 5-min intervals and analyzed for glucose. The livers were perfused as described in materials and methods. The data are reported as the mean ± SD of 6-8 individual liver perfusion experiments. AUC - area under the curves (µmol/g). *p < 0.05 *vs*. Normoglycemic group.

collected at 5-min intervals and the level of glucose was analyzed. The differences in the glucose production during (10-70 min) and before (0-10 min) the infusion of AGP permitted to calculated the area under the curves (AUC) which is expressed as µmol per gram. Moreover, in part of the liver perfusion experiments L-lactate, pyruvate and urea production were measured.

Metabolite concentrations were measured with standard enzymatic techniques: glucose was determined with glucose oxidase [12] (Gold Analisa® glucosae assay kit), L-lactate [14] and pyruvate [15] with lactate dehydrogenase and urea [16] (Gold Analisa® urea assay kit) was determined with urease. The concentration of ammonia was assayed by the same method used to measure urea, but without urease.

Determination of the maximal hepatic capacity to ammonia uptake and urea production from NH_4Cl

Ammonia uptake was measured by the difference between the concentration of NH_4Cl during and before the infusion of NH_4Cl. Liver perfusion experiments using increasing concentrations of NH_4Cl were performed. The addition of NH_4Cl in the perfusion fluid increased the rate of ammonia uptake until saturating concentration was reached (data not shown). The value obtained, *i.e.,* 2 mM, was used to verify if IIH influenced the hepatic capacity to ammonia uptake and urea production from ammonia.

Statistical analysis

Results are reported as means ± S.D. The program GraphPad Prism (4.02 version) was used to calculate the AUC. Data were analyzed statistically by the unpaired Student's *t*-test.

RESULTS

Hypoglycemic rats which received oral AGP (100 mg/kg) or ALA (100 mg/kg) showed higher (p < 0.05) blood levels of glucose than hypoglycemic animals that received oral saline (Fig. 2). However, blood levels of glucose after oral administration of GLN (100 mg/kg) was maintained similar as those observed in the hypoglycemic animals that received oral saline (Fig. 2).

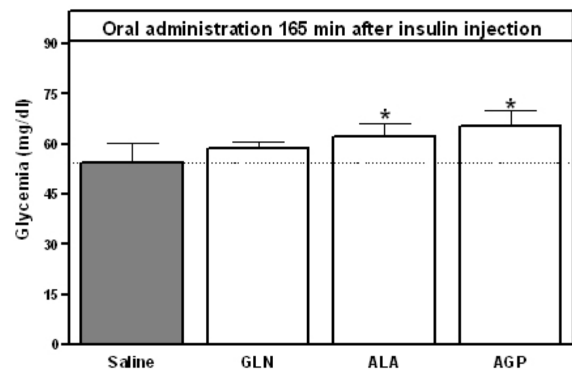

Figure 2. Effect of oral administration of saline (Hypoglycemic Control group), 100 mg/kg L-glutamine (GLN), 100 mg/kg L-alanine (ALA) or 100 mg/kg glutamine dipeptide (AGP) on glycemia in 24-h fasted rats. The substances were administered 165 min after insulin injection (IIH group) and glycemia was measured 15 min later (180 min). Data are reported as means ± SD of 6-8 animals. *p < 0.05 *vs*. Control group.

Figure 3 shows the AUC for glucose production during the infusion of saturating concentration of ALA (5 mM), GLN (5 mM) or AGP (5 mM). The saturating concentration represents the concentration in which the maximal glucose production was obtained to each gluconeogenic substrate. Thus, the AUC values, calculated by subtracting the basal rates (Fig. 1), indicated a more intense (P < 0.05) activation of glucose production in livers of IIH rats if compared with the values obtained from livers of normoglycemic rats (Control group) for each gluconeogenic substrate investigated.

In agreement with the results to glucose production (Fig. 3), livers from IIH rats which received saturating concentration of ALA or GLN produced higher (p < 0.05) amount of urea (Fig. 4), L-lactate (Fig. 5A) and pyruvate (Fig. 5B) if compared with livers of control normoglycemic

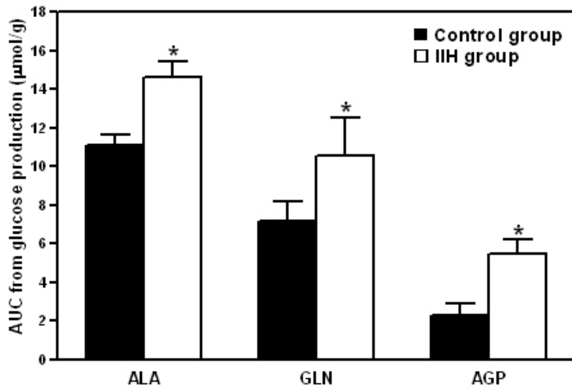

Figure 3. Glucose production from saturating concentration (5 mM) of L-alanine (ALA), L-glutamine (GLN) or glutamine dipeptide (AGP) in livers of hypoglycemic (IIH group, ❑) and normoglycemic (Control group, ■) 24-h fasted rats. The data are reported as means ± SD of 6-8 individual liver perfusion experiments. *p < 0.05 *vs*. Control group.

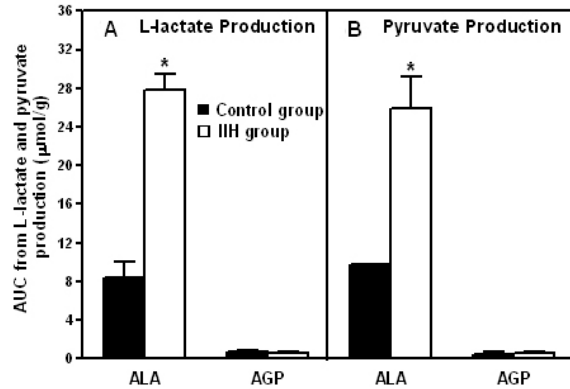

Figure 5. L-lactate production (A) and pyruvate production (B) from saturating concentration (5 mM) of L-alanine (ALA), or glutamine dipeptide (AGP) in livers of hypoglycemic (IIH group, ❑) and normoglycemic (Control group, ■) 24-h fasted rats. The data are reported as means ± SD of 6-8 individual liver perfusion experiments. *p < 0.05 *vs*. Control group.

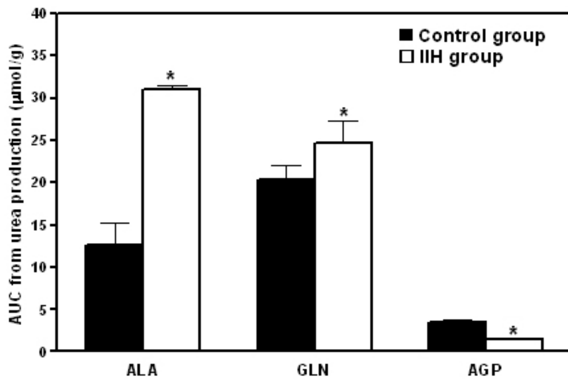

Figure 4. Urea production from saturating concentration (5 mM) of L-alanine (ALA), L-glutamine (GLN) or glutamine dipeptide (AGP) in livers of hypoglycemic (IIH group, ❑) and normoglycemic (Control group, ■) 24-h fasted rats. The data are reported as means ± SD of 6-8 individual liver perfusion experiments. *p < 0.05 *vs*. Control group.

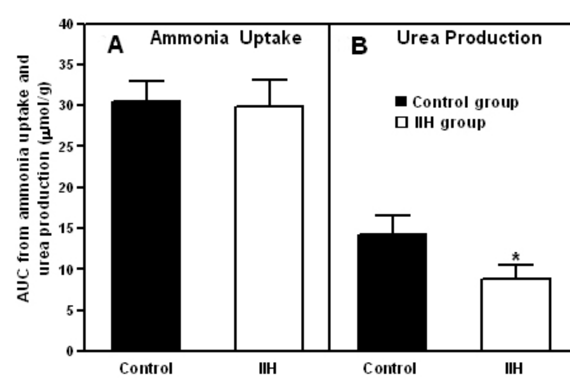

Figure 6. Ammonia uptake (**A**) and urea production (**B**) from saturating concentration (2 mM) of NH4Cl in livers of hypoglycemic (IIH group, ❑) and normoglycemic (Control group, ■) 24-h fasted rats. The livers were perfused as described in materials and methods. The data are reported as means ± SD of 6-8 individual liver perfusion experiments. *p < 0.05 *vs*. Control group.

rats. In contrast with ALA or GLN, livers from IIH rats which received saturating concentration of AGP produced lower ($p < 0.05$) amount of urea (Fig. 4) and similar production of L-lactate (Fig. 5A) and pyruvate (Fig. 5B) if compared with the values obtained from livers of control normoglycemic rats. Moreover, AGP showed lower glucose (Fig. 3), urea (Fig. 4), L-lactate (Fig. 5A) and pyruvate (Fig. 5B) production if compared with ALA.

Finally, livers from IIH rats which received saturating concentration of NH4Cl (2 mM) showed similar ammonia uptake (Fig. 6A) and lower urea production from ammonia (Fig. 6B) if compared with the values obtained from livers of control normoglycemic rats.

DISCUSSION

Our previous studies has been shown that normal rats should be a suitable model to study insulin induced hypoglycemia [3,4,13,17-22]. Thus by using normal rats submitted to long-term IIH we obtained acute glucose recovery with oral administration of AGP (Fig. 2). On the other hand, the absence of glycemia recovery with oral GLN could be ascribed to the fact that intestinal cells utilize this amino acid at high rates [5], reducing the amount available to the liver and other tissues. In agreement with these results, our recent studies also showed that oral administration of GLN did not promote acute glucose recovery during short term IIH [20].

To verify the participation of gluconeogenesis to glucose recovery promoted by AGP, livers from IIH and control normoglycemic rats were perfused with saturating concentration of AGP. This technique has the advantage of determining metabolite release rates directly from the effluent perfusate in the intact organ. Since AGP enters in the gluconeogenesis pathway after its degradation to ALA and GLN [6-10] these amino acids were investigated for comparative purpose.

Therefore, using a saturating concentration of AGP, ALA or GLN our results demonstrated an increased capacity to produce glucose from all these gluconeogenic substrates in livers from IIH rats when compared with the values obtained from livers of control normoglycemic rats (Fig. 3). In line with this observation the livers from IIH rats showed higher production of urea (Fig. 4), L-lactate (Fig. 5A) and pyruvate (Fig. 5B). The increased availability of L-lactate and pyruvate in the hepatocyte favored gluconeogenesis [20] and helps to explain the largest hepatic glucose production in livers from IIH group.

The higher urea production (Fig. 4) occurred in spite of the decreased ureagenic capacity showed by livers of IIH rats (Fig. 6B) reinforcing the idea that the increased urea production during the infusion of ALA or GLN was consequence of the increased catabolism of these ammonia precursors. Thus, we concluded that the hepatic glucose production is a priority in a condition where the availability of glucose to the brain must be maintained.

The mechanism involved in the increased capacity to produce glucose under long- term IIH[23] probably also involves increased release of counterregulatory hormones [21], particularly glucagon, considering that not only IIH stimulates the release of this hormone but also the administration of AGP [10].

In spite the fact that AGP and ALA promote the same degree of glucose recovery, the hepatic production of glucose, urea, pyruvate and L-lactate from AGP was lower than ALA. From these results we can suggest that the effect of AGP on glycemia recovery was not restricted to the intensification of the hepatic glucose production. In agreement with this suggestion AGP increased the availability of ALA and GLN as metabolic fuel to several extra hepatic tissues including the kidneys where GLN is the main gluconeogenic substrate [24].

Thus, we can conclude that the oral administration of AGP promote acute glucose recovery in rats submitted to long-term IIH with less intense participation of liver metabolism, particularly hepatic gluconeogenesis.

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