Oral Glutamine Dipeptide Prevents against Prolonged Hypoglycemia Induced by Detemir Insulin in Rats

Antonio Machado Felisberto-Júnior, a Fernando Canas Manso, a
Vilma Aparecida Ferreira de Godoi Gazola, b Simoni Obici, a Sandonaid Andrei Geisler, a and
Roberto Barbosa Bazotte*. a

a Department of Pharmacy and Pharmacology, State University of Maringá; and b Department of Morphophysiological Sciences, State University of Maringá; Maringá, PR 87020–900, Brazil.

Received August 26, 2008; accepted October 31, 2008; published online November 13, 2008

The role of glutamine dipeptide (GDP) to prevent against prolonged insulin induced hypoglycemia (IIH) in overnight fasted rats was investigated. The glycemia was measured 0, 2, 4, 8, and 10 h after an intraperitoneal (i.p.) injection (1 U/kg) of Detemir insulin. Because the lowest glycemia was obtained 4 h after insulin administration, the experiments were done at this time. The hypoglycemia obtained 4 h after insulin injection was partially prevented with increasing oral doses of GDP (1.56, 3.12, 6.25, 12.5 mg/kg). The best result was obtained with 12.5 mg/kg. However, from this dose (25.0, 50.0, 100.0 mg/kg), the values of glycemia progressively decreased (p<0.05). The effect of GDP to prevent prolonged IIH was mediated, partly at least, by an intensification of liver gluconeogenesis. Moreover, the increased portal availability of GDP, l-alanine and l-glutamine after GDP administration also contributed to the IIH prevention, since the rate of gluconeogenesis progressively augmented with the infusion of increasing concentrations of these substances. However, after getting the maximal value, the rate of liver gluconeogenesis decreased (p<0.05) if a more elevated concentration of l-alanine or l-glutamine was infused. On the other hand, the liver gluconeogenesis during the infusion of increasing concentrations of GDP was unchanged. Because, GDP did not directly inhibit liver gluconeogenesis, but an inhibition of this metabolic pathway was observed with low ammonia concentrations (from 0.062 mM) it is possible that the ammonia from the catabolism of GDP by extra hepatic tissues could inhibit liver gluconeogenesis. This mechanism could help to explain the lower glycemia obtained with more elevated doses of oral GDP.

Key words Detemir insulin; glutamine dipeptide; liver gluconeogenesis; hypoglycemia; rat

Most episodes of short-term symptomatic hypoglycemia are effectively treated by the ingestion of carbohydrates1) or glucagon injection.2) However, in spite the fact that glucose and glucagon are very effective to treat short term insulin-induced hypoglycemia (IIH), both antidotes show transitory effect1,2) and for this reason they are not effective to prevent prolonged IIH. Moreover, patients who receive insulin therapy frequently experience prolonged IIH, particularly nocturnal hypoglycemia that represents 55—75% of severe episodes of IIH.3) Therefore, for episodes of prolonged IIH during sleep, when the subject is unable to self-treat, new strategies to prevent IIH are necessary.3) However, there are few studies in the prevention of nocturnal hypoglycemia.5,6)

Because rats show a suitable experimental model to study hypoglycemia7—10) and considering the night habits of these animals, nocturnal IIH can be simulated with a diurnal pharmacological dose of Detemir insulin. Moreover, in spite the fact that glutamine dipeptide has been shown effective to promote acute glycemia recovery during long term IIH,10) its rule to prevent prolonged hypoglycemia was not investigated. Thus, by using this rat model7—11) we investigated if L-alanyl-l-glutamine peptide (glutamine catabolism stimulates the release of glucagon.16,17)

In addition the contribution of the liver gluconeogenesis from glutamine dipeptide and their metabolites l-alanine and l-glutamine to prevent prolonged hypoglycemia were investigated.

MATERIALS AND METHODS

Materials Detemir insulin (Levemir®) and glutamine dipeptide (Dipeptiven®) were purchased from Novo Nordisk and Fresenius, respectively. L-Alanine and L-glutamine were obtained from ICN Company. Food was represented by a commercial standard rodent chow (Nuvilab®) produced by Nuvital nutrients company (Colombo, Paraná State, Brazil).

Animals Adult male Wistar rats (180—220 g) were maintained on food and water ad libitum before the initiation of experimental procedures. The manipulation of the animals was approved by the ethical committee of the State University of Maringá, PR, Brazil (approval number 042/2006). On the day before the experiment the animals were food deprived from 5:00 p.m. All experiments were performed with overnight fasted rats (5:00 p.m.—9:00 a.m.).

Experimental Prolonged IIH A preliminary experiment to characterize the prolonged IIH after an intraperitoneal (i.p.) injection of Detemir insulin (1.0 U/kg) was done. Detemir insulin was not diluted but intraperitoneally injected (9:00 a.m.) with help of an infusion pump (Insight®). Blood was obtained by decapitation. The values obtained for glycemia at 0, 2, 4, 6, 8 and 10 h (means±S.D., n=4) after the injection of Detemir insulin were 95.3±3.4 mg/dl,
50.4 ± 4.0 mg/dl, 28.1 ± 2.5 mg/dl, 44.9 ± 7.6 mg/dl, 68.2 ± 6.6 mg/dl, and 73.2 ± 1.2 mg/dl, respectively. From these data, we observed that glycemia decreased until 4 h after insulin injection (phase of decreasing glycemia) and then progressively increased (phase of glycemia recovery). Thus, to verify whether oral glutamine dipeptide promotes glycemia prevention, this substance was administered immediately after insulin injection and glycemia values were measured 4 h later, when the lowest value of glycemia was obtained.

**Effect of Oral (Gavave) Administration of Glutamine Dipeptide in the Prevention of Prolonged IIH**

The rats were killed by decapitation 4 h after simultaneous i.p. Detemir insulin injection plus oral glutamine dipeptide (0, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0, 100.0 mg/kg) and the blood was collected for serum glucose analysis.18)

**Liver Perfusion Experiments**

The rats were anesthetized with an i.p. injection of sodium thiopental (40 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* according to the protocol illustrated in the Figs. 1 and 2, in which after a pre-perfusion period (10 min), the gluconeogenic substrate was dissolved in the perfusion fluid, followed by a post-infusion period (10 min) to allow the return to basal levels. Samples of the effluent perfusion fluid were collected at 5-min intervals and the level of glucose18) and urea19) were analyzed. The differences in the glucose and urea production during and before the infusion of the gluconeogenic substrate permitted to calculate the area under the curves (*AUC*).

In the first set of experiments the glucose production from livers of rats that received an i.p. saline (normoglycemic rats) or i.p. Detemir insulin (hypoglycemic rats) were compared (Fig. 1).

In the second set of experiments livers from rats which received i.p. Detemir insulin (hypoglycemic rats) were infused with increasing concentrations of l-alanine, l-glutamine or glutamine dipeptide. Thus, the values of *AUC* shown in Figs. 3—5 were obtained by the difference between the glucose and urea production during and before the infusion of the gluconeogenic substrates, as illustrated in Fig. 2A.

In the third set of experiments livers from rats which received i.p. Detemir insulin (hypoglycemic rats) were infused with l-lactate (3 mM) plus increasing concentrations of NH₄Cl. Thus, the values of *AUC* shown in Fig. 6 were obtained by the difference between the glucose and urea production during the infusion of l-lactate plus NH₄Cl (30—70 min) and the basal values (0—10 min), as illustrated in Fig. 2B.

---

**Fig. 1.** Glucose Production from Glutamine Dipeptide (5 mM) in Perfused Livers of Overnight Fasted Rats that Received an Intraperitoneal Injection (1 U/kg) of Detemir Insulin (Hypoglycemic, •) or Saline (Normoglycemic, □) 4h before the Liver Perfusion Experiment.

The effluent perfusate was sampled in 5 min intervals and analyzed for glucose. The *AUC*—areas under the curves (μmol/g) were obtained as described in Materials and Methods. Data were expressed as means±S.D. of 4 individual liver perfusion experiments. *p<0.05 vs. normoglycemic group.

**Fig. 2.** Demonstrative Experiment of Glucose Production from L-Alanine 5 mM, 10 mM and 15 mM (Panel A) or L-Lactate 3 mM Plus NH₄Cl 0.015 mM, 0.031 mM, 0.062 mM and 0.125 mM (Panel B) in Perfused Livers from Overnight Fasted Rats That Received an Intrapertoneal Injection (1 U/kg) of Detemir Insulin 4h before the Liver Perfusion Experiment.

The effluent perfusate was sampled in 5 min intervals and analyzed for glucose. The livers were perfused as described in Materials and Methods. *AUC*—areas under the curves (μmol/g).
Statistical analysis

Statistical analyses were performed with the software Graph Pad Prism 4. Data concerning glycemia were statistically analyzed by analysis of variance (ANOVA) followed by Tukey’s post-test. The results regarding liver perfusion experiments were analyzed by the unpaired Student’s t-test. Values are reported as mean values ± S.D. p values <0.05 was accepted for all comparisons.

RESULTS

Effect of Oral Glutamine Dipeptide on Glycemia at 4 h after Insulin Administration

As shown by Table 1 the hypoglycemia obtained 4 h after insulin injection was partially prevented with oral administration of glutamine dipeptide, since the values of glycemia increased (p<0.05) from 1.56 until 12.5 mg/kg (1.56, 3.12, 6.25, 12.5 mg/kg). On the other hand, from 12.5 mg/kg (25.0, 50.0, 100.0 mg/kg), the values of glycemia decreased (p<0.05).

Liver Perfusion Experiments

In the first set of experiments livers from rats that received an i.p. saline (normoglycemic rats) or i.p. Detemir insulin (hypoglycemic rats) were infused with glutamine dipeptide (5 mM). Livers from hypoglycemic rats showed higher (p<0.05) glucose production than livers of normoglycemic rats (Fig. 1).

In the second set of experiments livers from rats which received i.p. Detemir insulin (hypoglycemic rats) were infused with increasing concentrations of L-alanine, L-glutamine or glutamine dipeptide. The maximal hepatic glucose production to L-alanine (Fig. 3A), L-glutamine (Fig. 4A) and glutamine dipeptide (Fig. 5A) were obtained with 5.0 mM. From this value, the hepatic glucose production from L-alanine (Fig. 3A) or L-glutamine (Fig. 4A) decreased (p<0.05). However, to glutamine dipeptide the glucose production was maintained (Fig. 5A). On the other hand the urea production from L-alanine (Fig. 3B), L-glutamine (Fig. 4B) and glutamine dipeptide (Fig. 5B) did not decrease after getting the maximal value.
In the third set of experiments livers from rats which received i.p. Detemir insulin (hypoglycemic rats) were infused with L-lactate (3 mM) plus increasing concentrations of NH₄Cl (0.015, 0.031, 0.062, 0.125 mM). Thus, livers from IIH rats that received increasing concentrations of ammonia showed increasing values of urea production (Fig. 6B). On the other hand, the glucose production from L-lactate (Fig. 6A) decreased \( p<0.05 \) in the presence of NH₄Cl (from 0.062 mM).

**DISCUSSION**

Our previous study\(^9\) demonstrated that the blood concentrations of L-alanine and L-glutamine were decreased during long term IIH. In agreement with this study,\(^9\) we also demonstrated that oral glutamine dipeptide was able to promote glycemia recovery during long term IIH.\(^10\) Therefore, we expanded these previous studies investigating if glutamine dipeptide could prevent or decrease the intensity of prolonged IIH.

As shown by Table 1, the oral administration of glutamine dipeptide partially protects against prolonged hypoglycemia and the dose of 12.5 mg/kg represents the best result.

To investigate the participation of the hepatic gluconeogenesis in the protective effect of glutamine dipeptide against hypoglycemia, livers from rats submitted to prolonged IIH and normoglycemic rats were compared. The results demonstrated an increased \( p<0.05 \) glucose production from glutamine dipeptide in livers from IIH rats. The mechanism involved in the increased hepatic capacity to produce glucose under prolonged IIH probably involves increased release of counterregulatory hormones,\(^9\) which antagonise the effects of insulin on the key enzymes of gluconeogenesis.\(^9\) Another mechanism involves a favorable potential redox for gluconeogenesis, \( i.e. \), an increased reduced nicotinamide adenine dinucleotide/oxidized form of nicotinamide adenine dinucleotide (NADH/NAD\(^+\)) cytosolic ratio, inferred by the higher L-lactate/pyruvate ratio in livers from IIH rats.\(^8\)

Because it is hard to estimate exactly how much of the amount of L-alanine, L-glutamine and glutamine dipeptide actually entering in the liver, after the oral administration of glutamine dipeptide\(^9\) the effect of the portal infusion of increasing concentrations of these substances on liver glucose production was investigated. Thus, from experiments employing increasing concentrations of L-alanine (Fig. 3A), L-glutamine (Fig. 4A) or glutamine dipeptide (Fig. 5A) we observed that the increased availability of these gluconeogenic substrates also favored the liver glucose production and probably contributed to the hypoglycemia prevention promoted by increasing doses of oral glutamine dipeptide (from the dose of 1.56 mg/kg until 12.5 mg/kg). However, after getting the maximal hepatic glucose production, which reflects the liver capacity to produce glucose from the saturating concentration of L-alanine (Fig. 3A) or L-glutamine (Fig. 4A) the hepatic glucose production from these substances decreased \( p<0.05 \) if a more elevated concentration was used. In contrast, the hepatic glucose production from glutamine dipeptide was maintained (Fig. 5A).

Thus, a question can be raised: why the infusions of more elevated concentrations of L-alanine (Fig. 3A), L-glutamine (Fig. 4A), but not glutamine dipeptide (Fig. 5A) inhibited the liver gluconeogenesis? Before answering this question it is necessary to consider that the catabolism of amino acids generates ammonia which must be disposed of.\(^22\)

Therefore, if the glucose precursor is an amino acid, hepatic gluconeogenesis and ureagenesis, that share common intermediates, must be activated simultaneously.\(^23\) In agreement with this point of view the production of urea also increased \( p<0.05 \) during the infusion of increasing concentrations of L-alanine (Fig. 3B), L-glutamine (Fig. 4B) and glutamine dipeptide (Fig. 5B). However, the excess intramitochondrial concentration of ammonia obtained with the infusion of more elevated concentrations of L-alanine or L-glutamine probably decrease the intermediates of the citric acid cycle leading to depletion of ATP\(^24\) and consequently an inhibition of gluconeogenesis.

However, considering that glutamine dipeptide did not inhibit the hepatic glucose production, how can we explain the lower glycemia \( p<0.05 \) with more elevated oral doses (from 12.5 mg/kg until 100 mg/kg) of glutamine dipeptide? Firstly, it is necessary to consider that the participation of liver gluconeogenesis is not the whole story since glutamine dipeptide is an important energetic fuel to extra-hepatic tissues, particularly to the kidneys where glutamine is the main gluconeogenic substrate.\(^12\)

Therefore, the possibility of the inhibition of liver gluconeogenesis by ammonia from the catabolism of glutamine dipeptide by extra-hepatic tissues must be considered. Thus, to investigate the participation of ammonia in the inhibition of gluconeogenesis, the hepatic capacity in producing glucose from L-lactate (3 mM) during the infusion of increasing concentrations of ammonia in livers from IIH rats were evaluated. The reason to use L-lactate as gluconeogenic substrate was based in the following facts: 1) the liver catabolism of L-lactate does not produce ammonia; 2) L-lactate, which enters in the gluconeogenic pathway as pyruvate uses all steps of gluconeogenesis before producing glucose. Therefore, by using increasing concentration of ammonia we observed an inhibition of gluconeogenesis (Fig. 6A) with lower concentration of ammonia than that which gets the maximal ureagenesis (Fig. 6B).

Finally, the results shown here suggest that oral glutamine dipeptide could decrease the intensity of prolonged IIH. However, considering the possibility of the inhibition of liver gluconeogenesis and the reduction of glycemia with more elevated doses of glutamine dipeptide, the applicability of these results, with particular focus on the oral administration

| Detemir insulin + saline (Control group) | 15.4 ± 2.9 (9) |
| Detemir insulin + glutamine dipeptide (1.56 mg/kg) | 18.6 ± 5.5 (10) |
| Detemir insulin + glutamine dipeptide (3.12 mg/kg) | 23.5 ± 7.2 (10) |
| Detemir insulin + glutamine dipeptide (6.25 mg/kg) | 23.6 ± 5.9 (10) |
| Detemir insulin + glutamine dipeptide (12.5 mg/kg) | 41.9 ± 6.3* (7) |
| Detemir insulin + glutamine dipeptide (25.0 mg/kg) | 31.6 ± 6.3 (10) |
| Detemir insulin + glutamine dipeptide (50.0 mg/kg) | 20.0 ± 6.0 (10) |
| Detemir insulin + glutamine dipeptide (100 mg/kg) | 21.5 ± 6.4 (10) |

Glycemia were measured 4 h after the intraperitoneal injection of Detemir insulin. The values were expressed as means ± S.D. * vs other groups. ( ) = number of rats.
of this substance awaits further experimental studies.

Acknowledgments The authors are grateful to Carlos Eduardo de Oliveira and Solidalva Caruso de Oliveira for their technical assistance during the experiments. Research were supported by CNPq (grant number 472878/2006-2-Brazil).

REFERENCES