ANTONIO MACHADO FELISBERTO JUNIOR

INVESTIGAÇÃO DO EFEITO PROTETOR DA GLUTAMINA DIPEPTÍDEO NA HIPOGLICEMIA INDUZIDA POR INSULINA EM RATOS WISTAR: ESTUDOS *IN VIVO* E EM PERFUSÃO DE FÍGADO *IN SITU*

Prof. Dr. Roberto Barbosa Bazotte – Orientador

Universidade Estadual de Maringá
Departamento de Ciências Biológicas
Pós-Graduação em Ciências Biológicas
Área de Concentração Biologia Celular e Molecular

ANTONIO MACHADO FELISBERTO JUNIOR

INVESTIGAÇÃO DO EFEITO PROTETOR DA GLUTAMINA DIPEPTÍDEO NA HIPOGLICEMIA INDUZIDA POR INSULINA EM RATOS WISTAR: ESTUDOS *IN VIVO* E EM PERFUSÃO DE FÍGADO *IN SITU*

Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de Concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Mestre em Ciências Biológicas.

Maringá
2008

Dados Internacionais de Catalogação-na-Publicação
(CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

Felisberto Junior, Antonio Machado
F315i Investigação do efeito protetor da glutamina dipeptídeo na hipoglicemia induzida por insulina em ratos wistar: estudos *in vivo* e em perfusão de fígado *in situ* / Antonio Machado Felisberto Junior. -- Maringá : [s.n.], 2008.
30 f. : il.

Orientador : Prof. Dr. Roberto Barbosa Bazotte.
Dissertação (mestrado) - Universidade Estadual de Maringá, Programa de Pós-graduação em Ciências Biológicas, Área de concentração Biologia Celular e Molecular, 2008.

1. Hipoglicemia - Induzida por insulina. 2. Glicemia - Proteção. 3. Gliconeogênese - Metabolismo hepático. 4. GDP (glutamina dipeptídeo). I. Universidade Estadual de Maringá, Programa de Pós-graduação em Ciências Biológicas . II. Título.

CDD 21.ed.616.466

BIOGRAFIA

Antonio Machado Felisberto Junior nasceu em Curitiba, PR, em 17 de junho de 1982. Possui graduação em Ciências Biológicas pela Universidade Estadual do Oeste do Paraná colando grau em 2004. Deu prosseguimento a sua formação científica concluindo pós-graduação *lato sensu* em Ciências Morfofisiológicas, com enfoque em Corpo Humano pela Universidade Estadual do Oeste do Paraná no ano de 2006. Atualmente é mestrando na Universidade Estadual de Maringá, no curso de Ciências Biológicas, Área de concentração Biologia Celular e Molecular. Tem experiência na área de Biologia, com ênfase em Citologia, Biologia Celular e Bioquímica, atuando principalmente nos seguintes temas: Hipoglicemia Induzida por Insulina, Regulação da Glicemia e Metabolismo Hepático.

À Deus, minha família,

amigos e mestres.

AGRADECIMENTOS

À Deus.

Ao meu pai Antonio Machado Felisberto, minha mãe Marilene de Fátima Alves da Silva Felisberto e meu irmão Mariano Felisberto, meu avós Cezinato e Elza, tios, tias e primos pelo apoio, compreensão e constante estímulo.

Aos meus amigos, os quais por muitas vezes fizeram o papel de irmãos, mesmo nos momentos mais difíceis, Michael Lira, por me estimular a fazer a pós-graduação; Sandonaid Andrei Geisler, pelo auxílio de sempre na vida e no laboratório; Simoni Obici, por me ensinar diversos procedimentos laboratoriais e analíticos; Juliana K. Bilha, Felipe Almeida, Eduardo Hartmann, Carla, Claudecir e todos que de maneira direta ou indireta auxiliaram na realização deste trabalho.

Aos amigos do Laboratório Carlos Eduardo e Solidalva Oliveira, Gabriela e Thauany, pelo auxilio de sempre na realização e preparação dos longos e cansativos experimentos.

A Amanda Furjan Rial, pelo apoio, compreensão e estímulo, mesmo nos momentos mais difíceis, os quais foram fundamentais durante este longo percurso.

Aos mestres e amigos do programa de pós-graduação em Biologia Celular e Molecular.

E por último, mas não menos importante, ao meu orientador e amigo Professor Doutor Roberto Barbosa Bazotte, pela sempre presente orientação, não só quantos aos experimentos e resultados, mas também nos momentos difíceis que enfrentei neste período. Agradeço também pelo apoio e auxílio em um delicado momento de minha vida que foi o diagnóstico e início de tratamento do diabetes.

MUITO OBRIGADO!!!!!!

APRESENTAÇÃO

A presente dissertação de Mestrado foi redigida na forma de um artigo científico em consonância com as normas estabelecidas pelo Programa de Pós-graduação em Ciências Biológicas. O artigo científico, intitulado **"Oral glutamine dipeptide prevents against prolonged hypoglycemia induced by Detemir insulin in rats"**, foi redigido de acordo com as normas da revista ***Biological & Pharmaceutical Bulletin*.** Este estudo representa a continuidade dos trabalhos desenvolvidos pelo grupo de pesquisa do Laboratório de Farmacologia Endócrina que investigam os mecanismos de manutenção e recuperação da glicemia durante hipoglicemia induzida por insulina (HII). No presente trabalho, investigou-se em ratos Wistar, o efeito protetor da glutamina dipeptídeo na hipoglicemia de longa duração promovida pela administração de insulina Detemir. Além disso, a neoglicogênese e ureogêneses hepática a partir de L-alanina, L-glutamina ou glutamina dipeptídeo durante a HII foi investigada. Os resultados obtidos fornecem subsídios ao emprego de aminoácidos neoglicogênicos visando proteger pacientes submetidos à insulinoterapia em relação a episódios de hipoglicemia noturna.

Antonio Machado Felisberto-Junior, Roberto B. Bazotte. **Oral glutamine dipeptide prevents against prolonged hypoglycemia induced by Detemir insulin in rats.** *Biological & Pharmaceutical Bulletin*.

RESUMO GERAL

INTRODUÇÃO – Pacientes submetidos a insulinoterapia freqüentemente experimentam episódios de hipoglicemia induzida por insulina (HII) prolongada, em particular hipoglicemia noturna. Portanto, para episódios de HII durante o sono, quando o paciente está impossibilitado de se auto tratar, novas estratégias para prevenção desta condição são necessárias. Além disso, por serem animais noctivoros, ratos representam um modelo experimental adequado para o estudo da HII noturna, a qual pode ser simulada pela injeção diurna de uma dose farmacológica de insulina Detemir. Por outro lado, apesar de a glutamina dipeptídeo ter se mostrado efetiva na recuperação aguda da glicemia durante HII de longa duração, seu efeito protetor contra a HII prolongada não foi investigada. Assim, empregando modelo experimental de HII noturna investigou-se o papel da glutamina dipeptídeo em prevenir ou reduzir a intensidade da HII prolongada. **MÉTODOS –** Utilizou-se ratos machos Wistar (180-220 g). Estes foram privados de alimentos a partir das 17:00 h, recebendo insulina Detemir no dia seguinte às 8:00 h. O protocolo experimental utilizado foi aprovado pelo Comitê de Ética em Pesquisa Animal (n° 042/2006). Um experimento preliminar para caracterização da HII prolongada após a injeção intraperitoneal (ip) de insulina Detemir (1,0 U/kg) foi realizado. Uma vez estabelecido o tempo de 4 h após a injeção de insulina Detemir, como o de menor glicemia, este tempo foi selecionado para os experimentos *in vivo* e de perfusão de fígado *in situ*. Nos experimentos *in vivo* a injeção ip de insulina Detemir foi realizada simultaneamente com a administração intragástrica de doses crescentes de glutamina dipeptídeo (1,56; 3,12; 6,25; 12,5; 25,0; 50,0 ou 100,0 mg/kg). Em seguida, amostras de sangue, para avaliação da glicemia foram coletadas 4 h após a administração simultânea de insulina Detemir e de glutamina dipeptídeo. Para os experimentos em fígado *in situ*, os ratos foram anestesiados com injeção ip de tiopental (45 mg/kg) e em seguida submetidos a laparotomia. Os fígados foram perfundidos *in situ*

utilizando solução de Krebs-Hanseleit bicarbonato tamponado (pH 7,4) e saturado com O_2/CO_2 (95%/5%), além dos substratos gliconeogênicos. O perfusado foi coletado a cada 5 min, as concentrações de glicose e uréia analisadas e a partir dos valores obtidos avaliou-se a gliconeogênese e ureogênese, respectivamente. Para verificação da influência da concentração de amônia na gliconeogênese hepática, crescentes concentrações de NH_4Cl foram empregadas e seu efeito na produção de glicose a partir de L-lactato (3 mM) foi investigada. **RESULTADOS E DISCUSSÃO –** A administração oral de glutamina dipeptídeo protege parcialmente contra HII prolongada, sendo que a dose de 12,5 mg/kg representa o melhor resultado. Por outro lado, considerando que é difícil estimar a quantidade de L-alanina, L-glutamina ou glutamina dipeptídeo administrada que alcança a veia porta, o efeito da infusão portal de crescentes concentrações destas substâncias sobre a produção hepática de glicose foi investigada. Assim, em experimentos empregando concentrações crescentes de L-alanina, L-glutamina ou glutamina dipeptídeo observou-se que a crescente disponibilidade destas substâncias favorece a produção hepática de glicose e poderia explicar, pelo menos parcialmente, a melhor recuperação da glicemia promovida pelo emprego de crescentes doses orais de glutamina dipeptídeo. Porém, após alcançar a produção máxima de glicose, esta diminui ($P<0,05$) se concentrações mais elevadas de L-alanina ou L-glutamina são empregadas. Entretanto, para a glutamina dipeptídeo a produção hepática de glicose foi mantida, o que nos leva a levantar a seguinte questão: porque a infusão de altas concentrações de L-alanina ou L-glutamina, mas não de glutamina dipeptídeo, inibe a gliconeogênese? Antes de responder esta questão é necessário considerar que o catabolismo de aminoácidos gera amônia, a qual necessita ser eliminada. Portanto, se o precursor de glicose é um aminoácido, a gliconeogênese e a ureogênese, que possuem intermediários comuns, são ativadas simultaneamente. De acordo com este ponto de vista a produção de uréia também aumenta ($P<0,05$) durante a infusão de crescentes concentrações

de L-alanina, L-glutamina e glutamina dipeptídeo. Todavia, o excesso de concentração intramitocondrial de amônia obtido com a infusão de mais elevadas concentrações de L-alanina ou L-glutamina diminuem os intermediários do ciclo do ácido cítrico (TCA), acarretando redução do ATP e consequentemente inibição da gliconeogênese. Porém, como a glutamina dipeptídeo não inibe a produção hepática de glicose, como poderíamos explicar a relação inversa entre a dose oral de glutamina dipeptídeo e os valores da glicemia? Primeiro é preciso considerar que além da participação na gliconeogênese hepática, a glutamina dipeptídeo também constitui importante fonte energética para tecidos extra-hepáticos, particularmente os rins onde a glutamina é o principal substrato gliconeogênico. Portanto, a possibilidade de inibição da gliconeogênese hepática pela amônia a partir do catabolismo da glutamina dipeptídeo em tecidos extra-hepáticos precisa ser considerada. Assim, para investigar a participação da amônia na inibição da gliconeogênese, a produção de glicose a partir de L-lactato durante a infusão de crescentes concentrações de amônia no fígado proveniente de ratos HII foi avaliadas. O L-lactato foi empregado pelas seguintes razões: 1) o catabolismo hepático do L-lactato não produz amônia; 2) o L-lactato utiliza todas as etapas da gliconeogênese antes de produzir glicose. Assim, empregando concentrações crescentes de amônia observou-se inibição da gliconeogênese com concentrações de amônia inferiores à necessária para se alcançar o valor máximo de ureogênese. **CONCLUSÕES –** os resultados sugerem que a administração oral de glutamina dipeptídeo reduziria a intensidade da HII prolongada. Porém, considerando a possibilidade redução da glicemia com doses mais elevadas de glutamina dipeptídeo, a aplicabilidade destes resultados necessita de um maior volume de estudos empregando este modelo experimental de HII.

GENERAL ABSTRACT

INTRODUCTION – Patients who receive insulin therapy frequently experience prolonged insulin induced hypoglycemia (IIH), particularly nocturnal hypoglycemia that represents 55-75% of severe episodes of hypoglycemia. Therefore, for hypoglycemic episodes during sleep, when the subject is unable to self-treat, new strategies to prevent this condition are necessary. Because rats show a suitable experimental model to study IIH and considering the night habits of these animals, nocturnal IIH can be simulated with a diurnal pharmacological dose of Detemir insulin. On the other hand, in spite the fact that glutamine dipeptide has been shown effective to promote acute glucose recovery during long term IIH, its rule to prevents prolonged hypoglycemia was not investigated. Thus, by using an experimental model of nocturnal IIH we investigated if glutamine dipeptide could prevent or decrease the intensity of prolonged IIH. **METHODS –** Male Wistar (180-220 g) rats were used. The food was withdrawn 5:00 p.m and Detemir insulin was injected in the next day at 8:00 a.m. The experimental protocol was approved by the Institutional Animal Welfare Committee (number 042/2006). A preliminary experiment to characterize the prolonged IIH after an intraperitoneal (ip) injection of Detemir insulin (1.0 U/kg) was done. Since IIH was well established 4 h after insulin injection, this time was selected for *in vivo* and *in situ* liver perfusion experiments. To *in vivo* experiments an ip injection of Detemir insulin (1.0 U/kg) was done with simultaneous intragastric administration of increasing doses of glutamine dipeptide (1.56, 3.12, 6.25, 12.5, 25.0, 50.0 or 100.0 mg/kg). The blood samples to evaluate glycemia, obtained by decapitation, were collected 4 h after the simultaneous administration of Detemir insulin and glutamine dipeptide. For *in situ* liver perfusion experiments the rats were anesthetized by an ip injection of thiopental (45 mg/kg) 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%) and gluconeogenic substrates. Samples of the effluent perfusion fluid were collected at 5 min intervals and the glucose and urea concentration was analyzed. The differences in the glucose and urea production during and before the infusion of the gluconeogenic substrate allowed calculating gluconeogenesis and ureagenesis, respectively. To verify the influence of ammonia concentration on liver gluconeogenesis, increasing concentrations of NH_4Cl were employed and their effect on glucose production from L-lactate (3 mM) was investigated.

RESULTS AND DISCUSSION – The oral administration of glutamine dipeptide partially protects against prolonged hypoglycaemia and the dose of 12.5 mg/kg represents the best result. On the other hand, because it is hard to estimate exactly the amount of L-alanine, L-glutamine and glutamine dipeptide actually entering in the portal vein, after the administration of glutamine dipeptide, the effect of the portal infusion of increasing concentrations of these substances on liver glucose production was investigated. Thus, from experiments employing increasing levels of L-alanine, L-glutamine or glutamine dipeptide we observed that the increased availability of these gluconeogenic substrates favored the liver glucose production and could explain, at least partially, the better glucose recovery by using increasing doses of oral glutamine dipeptide. However, after getting the maximal hepatic glucose production, the liver glucose production decreased ($P<0.05$) if a more elevated concentration of L-alanine or L-glutamine was infused. In contrast, the hepatic glucose production from glutamine dipeptide was maintained. Thus, a question can be raised: why the infusion of more elevated concentrations of L-alanine, L-glutamine, but not glutamine dipeptide inhibited gluconeogenesis? Before answering this question it is necessary to consider that the catabolism of amino acids generates ammonia which must be disposed of. Therefore, if the glucose precursor is an amino acid, hepatic gluconeogenesis and ureagenesis, that share common intermediates, must be activated simultaneously. In

agreement with this point of view the production of urea also increased ($P<0.05$) during the infusion of increasing levels of L-alanine, L-glutamine and glutamine dipeptide. However, the excess intramitochondrial concentration of ammonia obtained with the infusion of more elevated concentration of L-alanine or L-glutamine decreased the intermediates of TCA cycle leading to depletion of ATP and consequently an inhibition of gluconeogenesis. However, considering that glutamine dipeptide did not inhibited hepatic glucose production, how can we explain the inverse relationship between the oral dose of glutamine dipeptide and the value of glycemia? 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. 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 during the infusion of increasing concentrations of ammonia in livers from IIH rats were evaluated. The reason to use L-lactate was based in the following facts: 1) the liver catabolism of L-lactate does not produce ammonia; 2) L-lactate uses all steps of gluconeogenesis before producing glucose. Thus, by using increasing concentration of ammonia we observed an inhibition of liver gluconeogenesis with lower concentration of ammonia than that necessary to get the maximal ureagenesis. **CONCLUSION**. Our previous results and the data shown here suggest that oral glutamine dipeptide could decrease the intensity of prolonged IIH. However, considering the possibility of the reduction of glycemia with more elevated doses of glutamine dipeptide, the applicability of these results, with particular focus to the oral administration of this substance await further studies with this experimental model.

Biological & Pharmaceutical Bulletin

Regular Article
Pharmacology

Oral glutamine dipeptide prevents against prolonged hypoglycemia induced by Detemir insulin in rats

Antonio Machado FELISBERTO-JUNIOR,[a] Fernando Canas MANSO,[a] Vilma A.F.G GAZOLA, [b] Simoni OBICI, [a] Sandonaid Andrei GEISLER, [c] and Roberto Barbosa BAZOTTE [*, a]

[a] *Department of Pharmacy and Pharmacology, State University of Maringá, Maringá, PR 87020-900, Brazil:* [b] *Department of Morphophysiological Sciences, State University of Maringá, Maringá, PR 87020-900, Brazil:*

*** To whom correspondence should be addressed.**

Roberto Barbosa Bazotte, Ph.D., Department of Pharmacy and Pharmacology, State University of Maringá, Maringá, PR 87020-900, Brazil
Fax: +55-44-3261-4999. E-mail: rbbazotte@uem.br

Running title: *Glutamine dipeptide prevents against hypoglycemia*

Oral glutamine dipeptide prevents against prolonged hypoglycemia induced by Detemir insulin in rats

The role of glutamine dipeptide (GDP) to prevents 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 (ip) 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 and 12.5 mg/kg). The best result was obtained with 12.5 mg/kg. However, from this dose (25.0, 50.0 and 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 were unchanged. Because, GPD 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; hepatic gluconeogenesis; hypoglycemia; rat

Most episodes of short-term symptomatic hypoglycemia are effectively treated by the ingestion of carbohydrates[1)] 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 effect[1,2)] 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.[4)] However, there are few studies in the prevention of nocturnal hypoglycemia.[5,6)]

Because rats show a suitable experimental model to study hypoglycemia [7-10)] 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, [11)] its rule to prevent prolonged hypoglycemia was not investigated. Thus, by using this rat model [7-11)] we investigated if L-alanyl-L-glutamine peptide (glutamine dipeptide) could help against prolonged IIH.

The choice of glutamine dipeptide was based in the following facts: 1) glutamine dipeptide results of the combination of the most abundant blood amino acid, i.e., L-glutamine[12)] and the most important liver glucose precursor, i.e., L-alanine, [13)] 2. very high doses of oral glutamine dipeptide did not show acute or subchronic toxicity,[14)] 3) glutamine dipeptide overcomes the intestinal catabolism of L-glutamine,[15)] 4) L-alanine from glutamine dipeptide 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 (ip) 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[18] 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 them progressively increased (phase of glycemia recovery). Thus, to verify whether oral glutamine dipeptide promote 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 (gavage) administration of glutamine dipeptide in the prevention of prolonged IIH The rats were killed by decapitation 4 h after simultaneous ip Detemir insulin injection plus oral glutamine dipeptide (0, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0 and 100.0 mg/kg) and the blood was collected for serum glucose analysis.[18)]

Liver perfusion experiments The rats were anaesthetized with an ip injection of sodium thiopental (40 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* according to the protocol illustrated in the Fig. 1 and Fig. 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 glucose[18)] and urea[19] were analyzed. The differences in the glucose and urea production during and before the infusion of the glucoenogenic substrate permitted to calculated the area under the curves (AUC).

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

In the second set of experiments livers from rats which received ip Detemir insulin (hypoglycemic rats) were infused with increasing concentrations of L-alanine, L-glutamine or glutamine dipeptide. Thus, the values of AUC shown in Fig. 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 ip Detemir insulin (hypoglycemic rats) were infused with L-lactate (3 mM) plus increasing concentrations of NH_4Cl. 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 NH_4Cl (30-70 min) and the basal values (0-10 min), as illustrated in Fig. 2B.

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 $\pm$ 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 ip saline (normoglycemic rats) or ip 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 ip 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 ip Detemir insulin (hypoglycemic rats) were infused with L-lactate (3 mM) plus increasing concentrations of NH_4Cl (0.015 mM, 0.031 mM, 0.062 mM, 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 NH_4Cl (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.[11)] 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 hypoglycaemia 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,[20)] which antagonise the effects of insulin on the key enzymes of gluconeogenesis.[21)] Another mechanism involves a favorable potential redox for gluconeogenesis, i.e., an increased $NADH/NAD^+$ 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 [15)] 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 prevetion 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 infusion of a more elevate 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 enter 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 of this substance await 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 supported by CNPq (grant number 472878/2006-2 - Brazil).

REFERENCES

1) Moore C., Woollard M., *Emerg. Med. J.*, **22**, 512–515 (2005).
2) Vermeulen M. J., Klompas M., Ray J. G., Mazza C., Morrinson J. L., *Diabetes Care*, **26**, 2472–2473 (2003).
3) Chase H.P., *J. Clin. Endocrinol. Metab.*, **91**, 2038–2039 (2007).
4) Davis S., Alonso M. D., *J. Diabetes Complications*, **18**, 60–68 (2004).
5) Saleh T.Y., Cryer P.E., *Diabetes Care*, **20**, 1231–1236 (1997).
6) Wiethop B. V., Cryer P. E., *Diabetes Care*, **16**, 1131– 1136 (1993).
7) Nascimento K. F., Garcia R. F., Gazola V. A. F. G., Souza H. M., Obici S., Bazotte R. B., *Life Science.*, **82**, 1018-1022 (2008).
8) Gazola V. A. F. G., Garcia R. F., Curi R., Pithon-Curi T. C., Mohamad M. S., Hartmann E. M., Barrena H. C., Bazotte R. B., *Cell Biochem. Funct.*, **25**, 211–216 (2007).
9) Garcia R. F., Gazola V. A. F. G., Barrena H. C., Hartmann E. M., Berti J., Toyama M. H., Boschero A. C., Carneiro A. M., Manso F. C., Bazotte R.B., *Amino Acids*, **33**, 151–155 (2007).
10) Sakamoto K., Yonoki Y., Fujioka T., Matsumura M., Mitsuta Y., Sano M., Saito M., Nakahara T., Ishii K., *Biol. Pharm. Bull.,* **29**, 574–576 (2006).
11) Garcia R. F., Gazola V. A. F. G., Curi R., Hartmann E. M., Barrena H. C., Nascimento K. F., Bazotte R. B. *Lat. Am. J. Pharm.*, **27**, 229-234 (2008).
12) Newsholme P., Lima M. M. R., Procopio J., Pithon-Curi T. C., Doi S. Q., Bazotte R. B., Curi R., Braz. *J. Med. Biol. Res.*, **36**, 153–163 (2003).
13) Bassoli B.K., Cassolla P., Borba-Murad G.R., Constantin J., Salgueiro-Pagadigorria C.L., Bazotte R.B, Silva R.S., Souza H.M. Cell Biochem Funct., **26**, 320-328 (2008).
14) Oda S, Mullaney T, Bowles A.J, Durward R, Lynch B, Sugimura Y. *Reg. Toxicol. Pharmacol*., **50**, 226-238, (2008).
15) Minami H., Morse E. L., Adibi A. S., *Gastroenterology*, **103**, 3–11 (1992).
16) Porcellati F, Pampanelli S, Rossetti P, Busciantella R. N, Marzotti S, Lucidi P, Santeusanio F, Bolli G. B, Fanelli C. G. *Diabetologia*, **50**, 422-430 (2007).
17) M'bemba J, Cynober L, de Bandt P, Taverna M, Chevalier A, Bardin C, Slama G, Selan J.L. *Diabetes Metabolism*, **29**, 412-417 (2003).
18) Bergmeyer H. U., Bernt E., "Determination of glucose with glucose-oxidase and peroxidase," ed. by Bergmeyer H. U., New York, 1974, pp. 1205–1215.
19) Gutmann I., Bergmeyer H.U., "Determination of urea, indicator reaction with phenol and hypochlorite," ed. by Bergmeyer H. U., New York, 1974, pp. 1790–1798.
20) Davis S. N., Dobbins R., Tarumi C., Jacobs J., Neal D., Cherrington A. D., *Am. J. Physiol.*, **268**, E521–E531 (1995).

21) Kraus-Friedmann, N., *Physiol. Rev.,* **64**, 170-259 (1984).
22) Comar, J. F., Suzuki-Kemmelmeyer, F., Nascimento, E. A., Bracht, A. *Am. J. Physiol.,* **293**, G838-849 (2007).
23) Newsholme EA, Leech AR., “Metabolism of Ammonia”, ed. by John Wiley & Sons., Chichester; 1983, pp. 481-508.

FIGURE LEGENDS

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, ⎫) 4 h before the liver perfusion experiments. 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 material and methods. Data were expressed as means ± SD 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 Δ (Panel A) or L-lactate 3 mM plus NH_4Cl 0.015 mM ●, 0.031 mM ⎡, 0.062 mM Δ and 0.125 mM (Panel B) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h 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).

Fig. 3. Glucose (A) and Urea (B) production from increasing concentrations of L-alanine (5 mM, 10 mM, and 15 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2A. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. L-alanine 10 mM.

Fig. 4. Glucose (A) and Urea (B) production from increasing concentrations of L-glutamine (5 mM, 10 mM, and 15 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2A. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. L-glutamine 2.5 mM.

Fig. 5. Glucose (A) and Urea (B) production from increasing concentrations of glutamine dipeptide (2.5 mM, 5 mM, 10 mM, and 20 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods section. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2A. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. glutamine dipeptide 2.5 mM.

Fig. 6. Glucose (A) and Urea (B) production from increasing concentrations of NH_4Cl (0.015 mM, 0031 mM, 0.062 mM, 0.125 mM) during the infusion of L-lactate (3 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion

experiment. The livers were perfused as described in Materials and Methods. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2B Data were expressed as means ± SD of 4 individual liver perfusion experiments. *$P < 0.05$ *vs*. NH_4Cl 0.062 mM.

Table 1. Blood glucose concentration (mg/dl) in overnight fasted rats which received intraperitoneal Detemir insulin simultaneously administered with oral saline (Control group) or increasing oral doses of glutamine dipeptide. Glycemia were measured 4 h after the intraperitoneal injection of Detemir insulin. The values were expressed as means ± SD. *P < 0.05 *vs*. other groups. ( ): number of rats.

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)

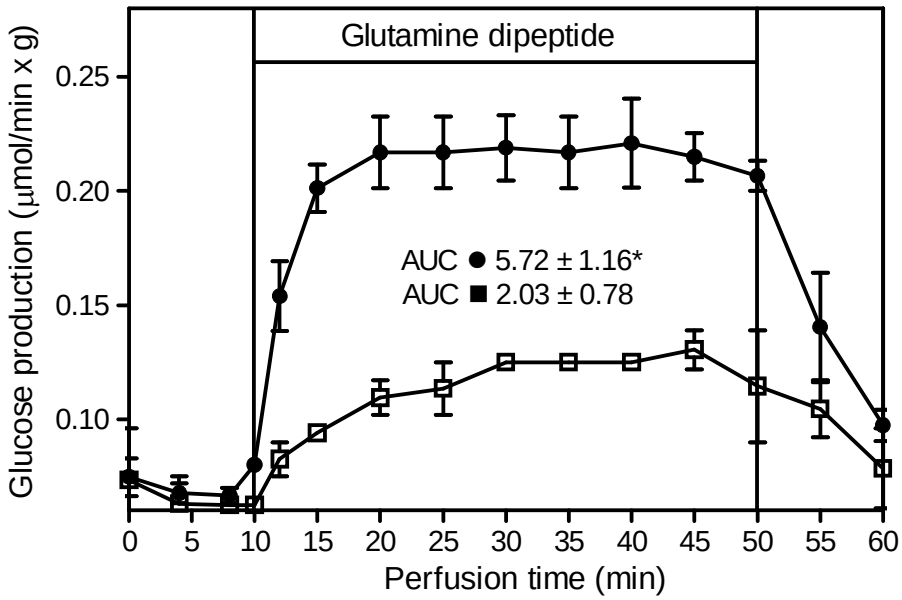

Fig. 1. Glucose production from glutamine dipeptide (5 mM) in perfused livers of overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin (hypoglycemic, ●) or saline (normoglycemic, ▯) 4 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals and analyzed for glucose. The AUC= areas under the curves (µmol/g) were obtained as described in Material and Methods. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. normoglycemic group.

Fig. 1.

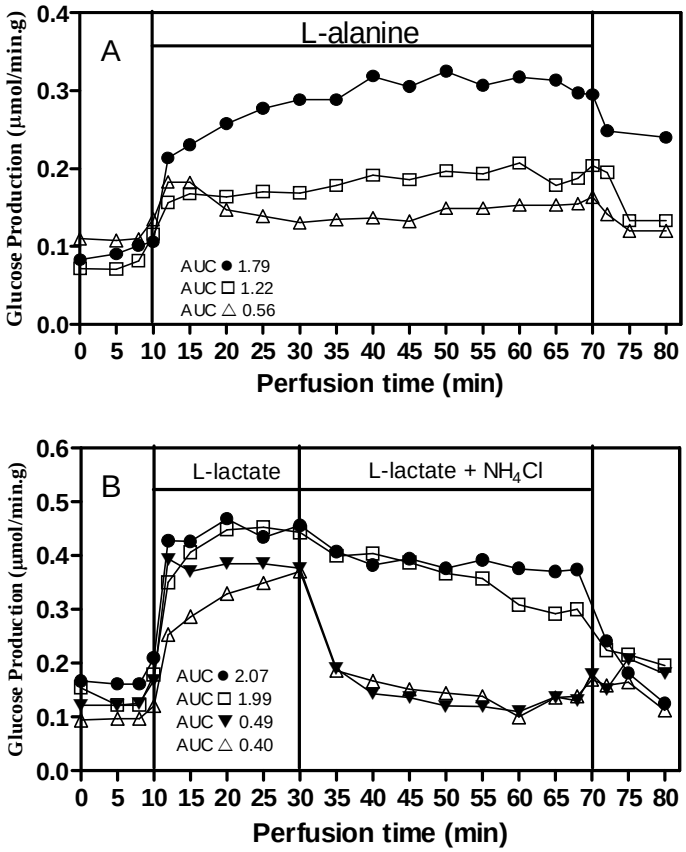

Fig. 2.

Fig. 2. Demonstrative experiment of glucose production from L-alanine 5 mM ●, 10 mM ⌈ and 15 mM Δ (Panel A) or L-lactate 3 mM plus NH4Cl 0.015 mM ●, 0.031 mM ⌈, 0.062 mM Δ and 0.125 mM (Panel B) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h 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).

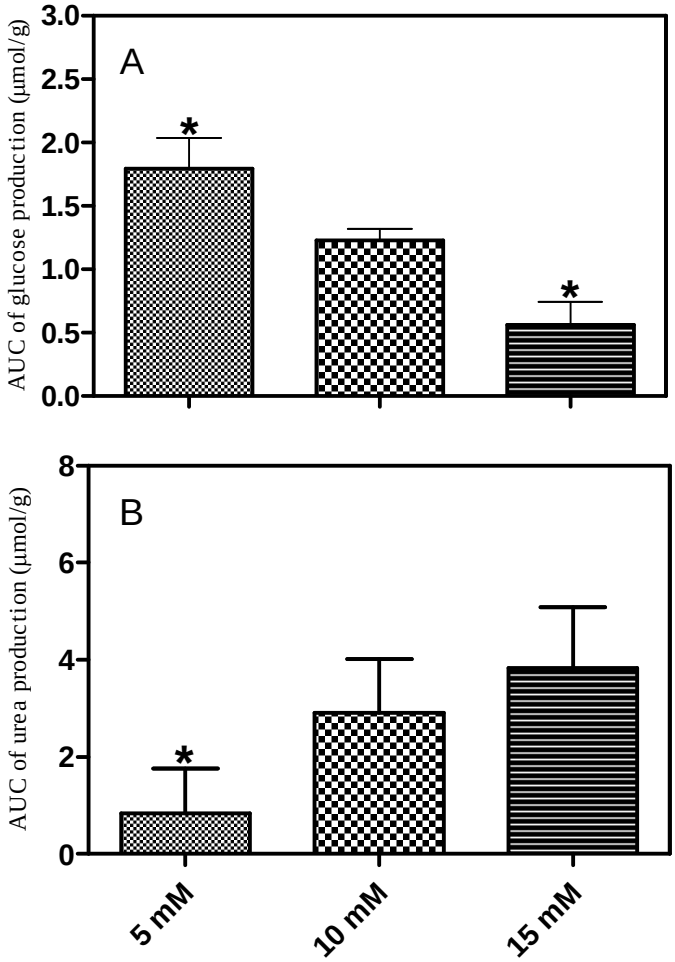

Fig. 3.

Fig. 3. Glucose (A) and Urea (B) production from increasing concentrations of L-alanine (5 mM, 10 mM, and 15 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2A. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. L-alanine 10 mM.

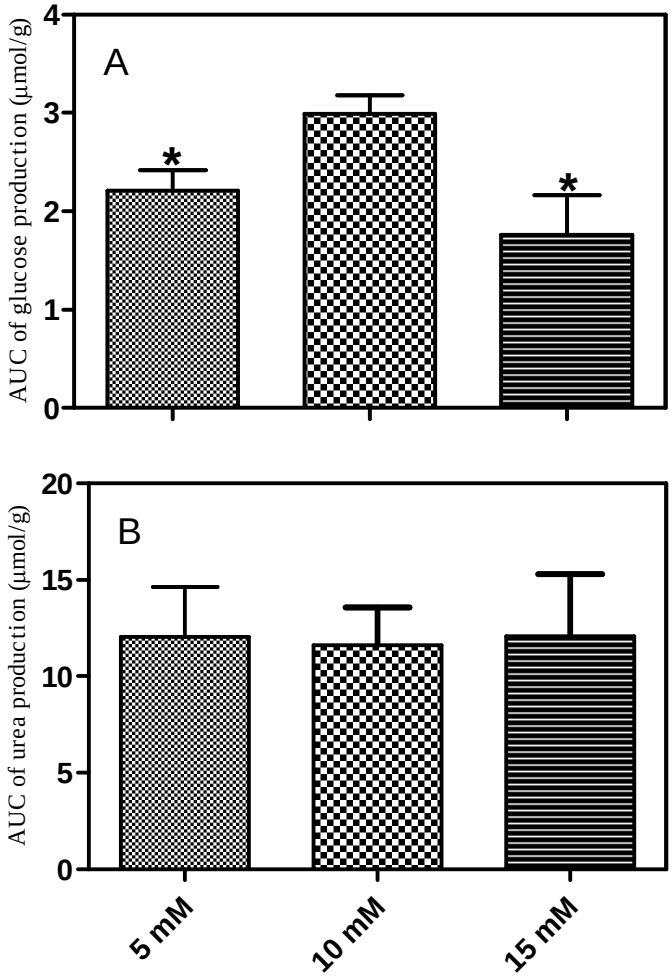

Fig. 4.

Fig. 4. Glucose (A) and Urea (B) production from increasing concentrations of L-glutamine (5 mM, 10 mM, and 15 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2A. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. L-glutamine 2.5 mM.

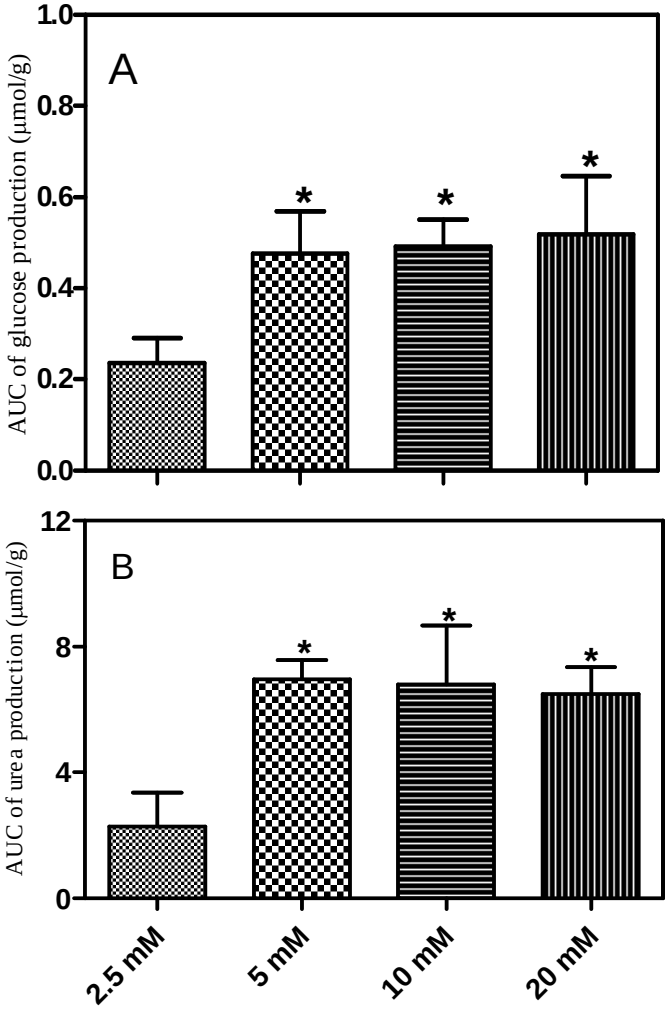

Fig. 5.

Fig. 5. Glucose (A) and Urea (B) production from increasing concentrations of glutamine dipeptide (2.5 mM, 5 mM, 10 mM, and 20 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods section. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2A. Data were expressed as means ± SD of 4 individual liver perfusion experiments. *P < 0.05 *vs*. glutamine dipeptide 2.5 mM.

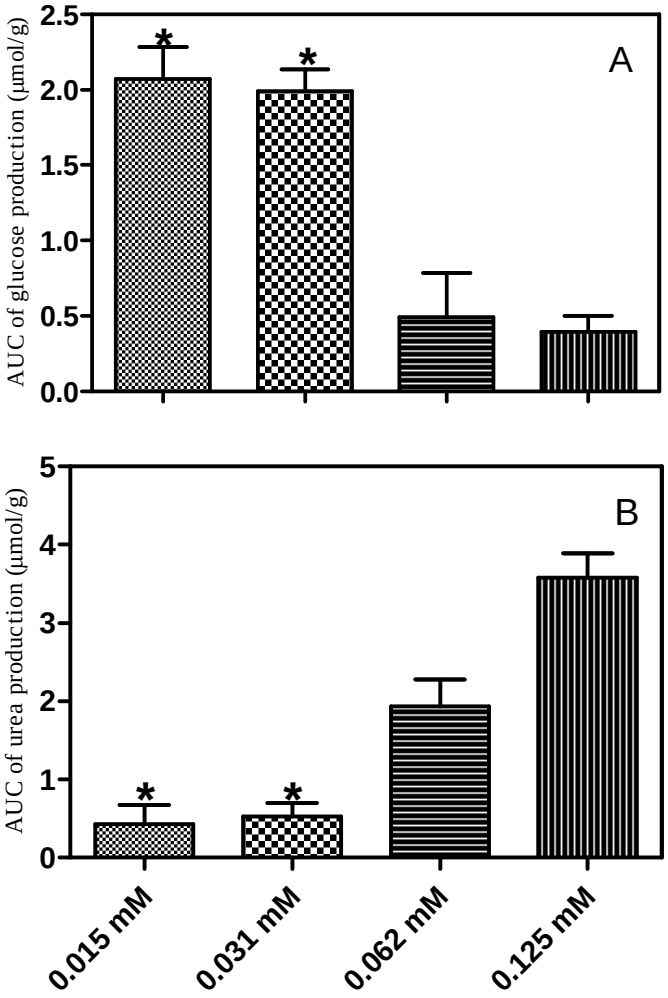

Fig. 6

Fig. 6. Glucose (A) and Urea (B) production from increasing concentrations of NH_4Cl (0.015 mM, 0031 mM, 0.062 mM, 0.125 mM) during the infusion of L-lactate (3 mM) in perfused livers from overnight fasted rats that received an intraperitoneal injection (1 U/kg) of Detemir insulin 4 h before the liver perfusion experiment. The livers were perfused as described in Materials and Methods section. The AUC= areas under the curves (µmol/g) were obtained as described in Fig. 2B Data were expressed as means ± SD of 4 individual liver perfusion experiments. *$P < 0.05$ *vs*. NH_4Cl 0.062 mM.

Livros Grátis

(http://www.livrosgratis.com.br)

Milhares de Livros para Download:

Baixar livros de Administração
Baixar livros de Agronomia
Baixar livros de Arquitetura
Baixar livros de Artes
Baixar livros de Astronomia
Baixar livros de Biologia Geral
Baixar livros de Ciência da Computação
Baixar livros de Ciência da Informação
Baixar livros de Ciência Política
Baixar livros de Ciências da Saúde
Baixar livros de Comunicação
Baixar livros do Conselho Nacional de Educação - CNE
Baixar livros de Defesa civil
Baixar livros de Direito
Baixar livros de Direitos humanos
Baixar livros de Economia
Baixar livros de Economia Doméstica
Baixar livros de Educação
Baixar livros de Educação - Trânsito
Baixar livros de Educação Física
Baixar livros de Engenharia Aeroespacial
Baixar livros de Farmácia
Baixar livros de Filosofia
Baixar livros de Física
Baixar livros de Geociências
Baixar livros de Geografia
Baixar livros de História
Baixar livros de Línguas