VILMA APARECIDA FERREIRA DE GODOI GAZOLA

EFEITO DA ADMINISTRAÇÃO DE L-ALANINA E L-GLUTAMINA NA RECUPERAÇÃO DA HIPOGLICEMIA INDUZIDA POR INSULINA DE CURTO PRAZO EM RATOS EM JEJUM: PARTICIPAÇÃO DA NEOGLICOGÊNESE E UREOGÊNESE HEPÁTICA

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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, área de concentração Biologia Celular, para obtenção do grau de Doutor em Ciências Biológicas.

Maringá, 2005

EFEITO DA ADMINISTRAÇÃO DE L-ALANINA E L-GLUTAMINA NA RECUPERAÇÃO DA HIPOGLICEMIA INDUZIDA POR INSULINA DE CURTO PRAZO EM RATOS EM JEJUM: PARTICIPAÇÃO DA NEOGLICOGÊNESE E UREOGÊNESE HEPÁTICA

Vilma Aparecida Ferreira de Godoi Gazola

ORIENTADOR: DR. ROBERTO BARBOSA BAZOTTE

À Deus:

por ser meu guia e minha luz,

por me acolher e me confortar,

por ser minha fonte de inspiração,

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APRESENTAÇÃO DO TRABALHO

Esta tese de Doutorado foi redigida na forma de DOIS ARTIGOS CIENTÍFICOS.

I. Vilma A F G GAZOLA[2], Rosângela F GARCIA[2], Eduardo M HARTMANN[3], Helenton C BARRENA[3], Helenir M SOUZA[4] ,Roberto B BAZOTTE[5] Comparative effects of isolated or combined oral administration of *l*-alanine and *l*-glutamine on glucose recovery during short term insulin induced-hypoglycemia[1]. Enviado para o periódico **Acta Pharmacologica Sinica**.

II. [1]V. A. F. G. Gazola, [1]R. F. Garcia, [2]R. Curi, [2]T. C. Pithon-Curi, [2]M. S. Mohamad, [1]E. M. Hartmann, [1]H. C. Barrena, [3]R. B. Bazotte. Acute effects of isolated and combined L-alanine and L-glutamine on hepatic gluconeogenesis, ureagenesis and glycaemia recovery in experimental short-term insulin induced hypoglycaemia. Enviado para o periódico **Cell Biochemistry and Function**.

ABSTRACT

Several clinical studies investigating thousands of patients demonstrated that the intensive insulin therapy prevent the development of the microvascular and macrovascular complications in diabetic patients. However, the rigorous glycemic control was associated with an increased incidence of insulin induced hypoglycemia (IIH), which is the major obstacle to the implementation of intensive treatment.

Insulin administration promote IIH, partly at least, by decreasing the mobilisation of glucose precursors to the liver. In addition, considering that during IIH the hepatic responsiveness to gluconeogenic precursors is maintained, the possibility of the administration of hepatic gluconeogenic precursors to promote glucose recovery (GR) must be considered. Therefore, since the first description of the treatment of hypoglycaemic symptoms with amino acids, about 40 years ago, studies in experimental animals, healthy volunteers and patients with diabetes have shown the possibility to use new antidotes instead glucose to treat IIH. Among others, favorable results were obtained with L-alanine, pyruvate, L-lactate and glutamine dipeptide. In agreement with these studies we observed that the intraperitoneal (ip) administration of L-Alanine (L-Ala) or L-Glutamine (L-Gln)

were more efficient than glucose itself to promote GR during IIH in rats. In addition, we found that the increased hepatic capacity to produce glucose from these amino acids was crucial to GR. But, it must be considered that these studies were done with 6 h-fasted rats, a condition where glycogenolysis contributed to hepatic glucose production (HGP). In contrast, the present work used 24-h fasted rats, a favourable condition for gluconeogenesis, in which the hepatic glycogen was completely depleted. Thus, the acute effect of isolated and combined oral administration (OA) of L-Ala and L-Gln on GR during short term IIH was investigated. Moreover, the participation of hepatic gluconeogenesis and ureagenesis to the GR were investigated.

The studies previously described resulted in two articles, which results and main conclusions will be presented separately.

Comparative effects of isolated or combined oral administration of L-alanine and L-glutamine on glucose recovery during short term insulin induced-hypoglycemia. The acute effects of isolated or combined OA of L-Ala and L-Gln on GR during short term IIH was compared. For this purpose glycemia from 24-h fasted rats that received ip regular insulin (IIH rats) or saline (COG rats) and 15 min later OA (100 $mg.Kg^{-1}$) of L-Ala, L-Gln, L-Ala + L-

Gln or glutamine dipeptide (GDP) were compared. Blood to measure glycemia was collected 15 min later, i.e., 30 min after insulin (1.0 $U.Kg^{-1}$) or saline injection. In addition, 30 min after insulin or saline injection, livers from IIH and COG rats were perfused with L-Ala, L-Gln, L-Ala + L-Gln or GDP and the maximal HGP were measured in all experiments. In part of the experiments the hepatic production of urea, L-lactate and pyruvate were measured. The results showed that differently of L-Gln, L-Ala + L-Gln or GDP, the OA of L-Ala promoted GR ($P<0.05$). In agreement, livers from IIH rats showed higher ($P<0.05$) maximal HGP from L-Ala if compared with L-Gln, L-Ala + L-Gln or GDP and this effect was obtained with 50% of the concentration used to obtain the maximal HGP in livers from COG rats. Thus we can conclude that isolated L-Ala showed the best GR. This effect was partly at least the consequence of the higher responsiveness of livers from IIH rats to produce glucose from L-Ala if compared with L-Gln, L-Ala + L-Gln or GDP.

Acute effects of isolated and combined L-alanine and L-glutamine on hepatic gluconeogenesis, ureagenesis and glycaemia recovery in experimental short-term insulin induced hypoglycaemia. The acute effects of isolated and combined L-Ala and L-Gln on liver gluconeogenesis, ureagenesis and GR during short-term IIH were investigated.

For this purpose, 24-h fasted rats that received ip regular insulin (1.0 U/Kg) or saline were compared. The studies were performed 30 min after insulin (IIH group) or saline (COG group) injection. Livers from IIH and COG groups were perfused with basal or saturating levels of L-Ala, L-Gln or L-Gln + L-Ala (L-G + L-A). The hepatic production of glucose, urea, L-lactate and pyruvate were markedly increased ($P < 0.001$) when the livers were perfused with saturating levels of L-Ala, L-Gln or L-G + L-A if compared with the basal levels of the same substrates. In addition, livers from IIH rats showed higher ability to produce glucose and urea from saturating levels of L-Ala if compared with L-Gln or L-G + L-A. In agreement with the results obtained in isolated livers, the OA of L-Ala (100 mg/Kg) promoted better GR than L-Gln (100 mg/Kg) or the combination of L-G (50 mg/Kg) + L-A (50 mg/Kg) in IIH rats. It can be concluded that L-Ala, but not L-Gln or L-G + L-A could help GR during IIH by a mechanism mediated, partly at least, due to the increased gluconeogenic and ureagenic efficiency.

RESUMO

Vários estudos clínicos investigando um grande número de pacientes demonstraram que a terapia intensiva com insulina evita o desenvolvimento de complicações micro e macro vasculares em pacientes diabéticos. No entanto, o rigoroso controle glicêmico foi associado com um aumento na incidência de hipoglicemia, que tornou-se o principal obstáculo à implementação do tratamento intensivo.

A administração de insulina promove hipoglicemia induzida por insulina (HII), parcialmente pelo menos, por reduzir a mobilização de precursores de glicose ao fígado. Além disso, considerando que durante a HII a responsividade hepática a precursores neoglicogênicos é mantida, a possibilidade de administração destes para promover a recuperação da glicemia (RG) pode ser considerada. Portanto, desde a primeira descrição do tratamento de sintomas hipoglicêmicos com aminoácidos, à cerca de quarenta anos, estudos em animais, voluntários saudáveis e pacientes com diabetes tem mostrado a possibilidade do uso de novos antídotos em vez de glicose para tratar HII. Entre outros, resultados favoráveis foram obtidos com L-alanina, piruvato, L-lactato e glutamina dipeptídeo. De acordo com estes estudos nós observamos que a administração intraperitoneal

(ip) de L-alanina (L-Ala) ou L-glutamina (L-Gln) foi mais eficiente que a própria glicose em promover RG durante HII em ratos. Além disso, nós observamos que o aumento na capacidade hepática em produzir glicose a partir desses aminoácidos foi crucial para a RG. Porém, devemos considerar que estes estudos foram feitos com ratos submetidos a jejum de 6 horas, uma condição onde a glicogenólise contribui com a produção hepática de glicose (PHG). Por outro lado, o presente trabalho usou ratos submetidos a jejum de 24 horas (J-24), uma condição favorável à neoglicogênese, em que o glicogênio hepático foi depletado. Assim, o efeito agudo da administração oral (AO) de L-Ala e L-Gln isoladas e/ou combinadas, sobre a RG durante HII de curto prazo foi investigado. Além disso, investigamos também a participação da neoglicogênese e ureogênese hepática na RG.

Os estudos previamente descritos resultaram em dois artigos, cujos resultados e principais conclusões são apresentados separadamente.

Efeito comparativo da administração oral de L-alanina e L-glutamina, isoladas ou combinadas, sobre a recuperação da glicemia durante hipoglicemia induzida por insulina de curto prazo. O efeito agudo da AO de L-Ala e L-Gln, isoladas ou combinadas, sobre a RG durante a HII de curto prazo foi comparada. Para este propósito a glicemia de

ratos J-24 que receberam injeção ip de salina (ratos COG) ou insulina regular (ratos HII) e 15 min depois a AO (100 mg.Kg^{-1}) de L-Ala, L-Gln, L-Ala + L-Gln ou glutamina dipeptídeo (GDP) foi comparada. O sangue utilizado para dosar a glicemia foi coletado 15 min após a AO, ou seja, 30 min após a injeção de salina ou insulina (1,0 U.Kg^{-1}). Além disso, 30 min após a injeção de salina ou insulina, fígados de ratos COG e HII foram perfundidos com L-Ala, L-Gln, L-Ala + L-Gln ou GDP e a PHG máxima foi avaliada. Em parte destes experimentos a produção hepática de uréia, L-lactato e piruvato foi quantificada. Os resultados mostraram que diferentemente de L-Gln, L-Ala + L-Gln ou GDP, a AO de L-Ala promoveu RG ($P < 0,05$). Da mesma forma, fígados de ratos HII mostraram maior ($P < 0,05$) PHG máxima a partir de L-Ala se comparada com L-Gln, L-Ala + L-Gln ou GDP, e este efeito foi obtido com 50% da concentração usada para obter a PHG máxima em fígados de ratos COG. Assim, podemos concluir que a L-Ala isolada mostrou melhor RG. E que este efeito foi, parcialmente pelo menos, conseqüência da maior responsividade dos fígados de ratos HII em produzir glicose a partir de L-Ala se comparado à L-Gln e às combinações.

Efeito agudo da L-alanina e L-glutamina sobre a neoglicogênese e ureogênese hepática e sobre a recuperação da glicemia durante hipoglicemia induzida

por insulina de curto prazo. O efeito agudo da L-Ala e L-Gln sobre a neoglicogênese e ureogênese hepática e sobre a RG durante hipoglicemia induzida por insulina de curto prazo foi investigada. Para este propósito, ratos J-24 que receberam injeção ip de salina ou insulina (1,0 $U.Kg^{-1}$) foram comparados. Os estudos foram feitos 30 min após a injeção de salina (grupo COG) ou insulina (grupo HII). Os fígados dos grupos COG e HII foram perfundidos com níveis basais ou saturantes de L-Ala, L-Gln ou L-Gln + L-Ala (L-G + L-A). A produção hepática de glicose, uréia, L-lactato e piruvato foi marcadamente aumentada ($P < 0,001$) quando os fígados foram perfundidos com concentrações saturantes de L-Ala, L-Gln ou L-G + L-A se comparado com os níveis basais dos mesmos substratos. Além disso, fígados de ratos HII mostraram maior habilidade de produzir glicose e uréia a partir de concentrações saturantes de L-Ala se comparados com L-Gln or L-G + L-A. De acordo com os resultados obtidos em fígados isolados, a AO de L-Ala (100 mg/Kg) promoveu melhor RG que L-Gln (100 mg/Kg) ou a combinação de L-G (50 mg/Kg) + L-A (50 mg/Kg) em ratos HII. Podemos então concluir que L-Ala, porém não L-Gln ou L-G + L-A, poderia auxiliar na RG, durante HII, por um mecanismo mediado, parcialmente pelo menos, pelo aumento na eficiência neoglicogênica e ureogênica.

----------**ARTIGO I**

“Comparative effects of isolated or combined oral administration of *l*-alanine and *l*-glutamine on glucose recovery during short term insulin induced-hypoglycemia”

Comparative effects of isolated or combined oral administration of *l*-alanine and *l*-glutamine on glucose recovery during short term insulin induced- hypoglycemia[1]

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ABSTRACT

AIM: To compare the acute effects of isolated or combined oral administration (OA) of *l*-alanine (*l*-ala) and *l*-glutamine *(l*-gln) on glucose recovery (GR) during short term insulin induced hypoglycemia (IIH). **METHODS**: glycemia from 24 h fasted rats that received intraperitoneal (ip) regular insulin (IIH rats) or saline (COG rats) and 15 min later OA (100 $mg.Kg^{-1}$) of saline, glucose (glu), *l*-ala, *l*-gln, *l*-ala+*l*-gln or *l*-alanyl-*l*-glutamine (*l*-ala-*l*-gln) were compared. Blood to measure glycemia was collected 15 min later, i.e., 30 min after insulin (1.0 $U.Kg^{-1}$) or saline injection. In addition, 30 min after insulin or saline injection, livers from IIH and COG rats were perfused with *l*-ala, *l*-gln, *l*-ala+*l*-gln or *l*-ala-*l*-gln and the maximal hepatic glucose production (HGP) were measured in all experiments. In part of the experiments the hepatic production of urea, l-lactate and pyruvate were measured. **RESULTS**: Differently of glu, *l*-gln, *l*-ala+*l*-gln or *l*-ala-*l*-gln, the OA of *l*-ala promoted GR ($P<0.05$). In agreement, livers from IIH rats showed higher ($P<0.05$) maximal HGP from *l*-ala if compared with *l*-gln, *l*-gln+*l*-ala or *l*-ala-*l*-gln and this effect was obtained with 50% of the concentration used to obtain the maximal HGP in livers from COG rats. **CONCLUSION**: isolated *l*-ala showed the best

GR. This effect was partly the consequence of the higher efficiency of livers from IIH rats to produce glu from *l*-ala if compared with *l*-gln, *l*-ala+*l*-gln or *l*-ala-*l*-gln. **KEY WORDS** *l*-alanine; *l*-glutamine; gluconeogenesis; hypoglycemia; *l*-alanyl-*l*-glutamine.

INTRODUCTION

Glucose is used by virtually all cells in the body and gluconeogenesis is vital to the maintenance of a supply of glucose to the brain during insulin induced hypoglycemia (IIH)[1], particularly when the stores of the hepatic glycogen were depleted[2]. In agreement, our previous study[3] demonstrated that in spite of hyperinsulinemia the hepatic capacity to produce glucose from *l*-alanine and *l*-glutamine was increased during IIH. Moreover, these findings give the possibility for using *l*-alanine and/or *l*-glutamine, instead glucose, to promote glucose recovery. In addition, we previously demonstrated that the combined administration of glucose precursors was more efficient than glucose itself to promote glucose recovery during IIH[4,5]. However, in these studies[4,5] glucose and glucose precursors were injected ip.

Since isolated ip administration of the most important gluconeogenic amino acid, i.e., *l*-alanine and the most

abundant blood amino acid, i.e., *l*-glutamine promoted better glucose recovery than glucose[5] we decided to compare the impact of oral administration of these amino acids[6,7] on glycemia during short term IIH. In addition the participation of hepatic gluconeogenesis to the glucose recovery was investigated.

Considering that glutamine is quantitatively the most important fuel for intestinal tissues[8] we decided to use not only isolated *l*-glutamine but also *l*-glutamine + *l*-alanine and *l*-alanyl-*l*-glutamine, a dipeptide more resistant than *l*-glutamine to intestinal metabolism[9] .

MATERIALS AND METHODS

Animals ♂ Wistar rats (*Rattus norvegicus*) weighing 180-220 g were starved (24 h) and divided in two groups. The IIH group was composed by rats, which received an ip injection of regular insulin (Novolin® R). Rats that received an ip injection of saline composed the control group (COG group). To minimize circadian variations all experiments started at 8:00 am. The manipulation of the animals followed the Brazilian law on the protection of animals.

Materials *l*-alany-*l*-lglutamine was purchased from Fresenius Kabi Brasil Ltda. *l*-alanine and *l*-glutamine were

obtained from ICN. Insulin (Novolin®) was purchased from Novo Nordisk (Brazil). All other reagents were of the highest purity obtainable.

Effect of gluconeogenic amino acids on glucose recovery A preliminary experiment to characterize the glycemic response to insulin was used to establish the lower glycemia after an ip injection of regular insulin (1.0 $U.Kg^{-1}$). The data showed that glycemia progressively decreased from a baseline value of 4.87 $mmol.L^{-1}$ (0 min) to 3.26 $mmol.L^{-1}$ (15 min), 2.29 $mmol.L^{-1}$ (30 min) and then progressively increased to 2.53 $mmol.L^{-1}$ (60 min), 2.75 (120 min) and 3.00 $mmol.L^{-1}$ (180 min). The values after saline injection (COG group) were respectively 4.87 $mmol.L^{-1}$ (0 min), 4.87 $mmol.L^{-1}$ (15 min), 4.84 $mmol.L^{-1}$ (30 min), 4.84 $mmol.L^{-1}$ (60 min), 5.05 (120 min) and 5.01 $mmol.L^{-1}$ (180 min). Because hypoglycemia was well established 15 min after insulin injection, this time was selected for oral administration of the amino acids. In addition, considering that the lower glycemia was obtained 30 min after insulin injection this time was selected to measure glycemia and gluconeogenesis.

Thus, *l*-alanine, *l*-glutamine, *l*-alanine + *l*-glutamine or *l*-alanyl-*l*-glutamine (100 $mg.Kg^{-1}$) were orally administered 15 min after insulin (IIH group) or saline (COG group) injection. Moreover, two additional subgroups which received oral saline

or glucose (100 mg.Kg^{-1}) 15 min after the insulin (IIH group) or saline (COG group) injection were included. Blood samples were collected by decapitation and glycemia was measured 15 min later, i.e., 30 min after insulin or saline injection (Fig 2).

Liver perfusion technique The rats were anaesthetized by ip injection of pentobarbital sodium (40 mg.Kg^{-1}) and submitted to laparotomy. The livers were perfused *in situ*[10] using Krebs Henseleit bicarbonate buffer, pH 7.4, saturated with a mixture of O_2/CO_2 (95/5%). The perfusion fluid was pumped through a temperature controlled (37ºC) membrane oxygenator prior to entering the liver via portal vein. The perfusion was performed in an open system with no recirculation of the perfusate[11]. A constant flow rate in each experiment was adjusted according to the liver weight (4 mL/g of tissue fresh weight per min).

Determination of the maximal hepatic capacity to produce glucose from *l*-alanine, *l*-glutamine, *l*-glutamine + *l*-alanine and *l*-alanyl-*l*-glutamine Liver perfusion experiments using increasing concentrations of *l*-alanine, *l*-glutamine and *l*-alanyl-*l*-glutamine were performed. The addition of these glucose precursors increased the rate of glucose production proportionately to the amount of precursor until saturating concentration is reached (data not shown).

The values obtained, (Tab 1), were used in the liver perfusion experiments.

Demonstrative experiment by using saturating concentration of *l*-glutamine (Fig 1) After a pre-perfusion period, (10 min), saturating concentration of *l*-glutamine was dissolved in the perfusion fluid and infused between the 10^{th} and 70^{th} min of the perfusion period, 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 levels of glucose[12] were analyzed. The differences in the glucose production during (10-70 min) and before (0-10 min) the infusion of *l*-glutamine permitted to calculated the area under the curve (AUC), expressed as $\mu mol.g^{-1}$. The AUCs of Fig 3, 4 and 5 were obtained from similar experiments. In addition, the same procedure was performed when *l*-alanine, *l*-glutamine + *l*-alanine and *l*-alanyl-*l*-glutamine were used as gluconeogenic substrates. Moreover, in part of the liver perfusion experiments *l*-lactate[13], pyruvate[14] and urea[15] production were measured.

Statistical analysis The results, including the AUC were analyzed by ANOVA or Student unpaired t-test, using the Graph-Pad Prism–version 6.0 program. The results are

presented as Mean ± SD. A 95% level of confidence ($P< 0.05$) was accepted for all comparisons.

RESULTS

IIH rats that received oral administration of *l*-alanine showed higher glycemia ($P<0.05$) than IIH rats that received saline, glucose, *l*-glutamine, *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine (Fig 2).

In agreement with these results, livers from IIH rats showed higher ($P<0.05$) glucose production (Fig 3) during the infusion of saturating concentration of *l*-alanine if compared with saturating concentration of *l*-glutamine, *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine (Tab 1). However, differently of *l*-glutamine, *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine the hepatic glucose production from *l*-alanine was lower ($P<0.05$) if we compared the results from IIH rats vs COG rats (Fig 3).

To understand why the hepatic catabolism of *l*-alanine, inferred by urea production, was increased ($P<0.05$) in livers from IIH rats (Fig 4) but glucose production (Fig 3) was decreased ($P<0.05$), the hepatic production of *l*-lactate and pyruvate from *l*-alanine was investigated. Thus, livers from IIH rats showed higher ($P<0.05$) *l*-lactate production and

lower ($P<0.05$) pyruvate production if we compared the results from IIH rats vs COG rats (Fig 5).

DISCUSSION

Our previous study[5] demonstrated that the administration of *l*-alanine and *l*-glutamine during short term IIH promoted better glucose recovery than glucose, opening the possibility to the administration of these amino acids in the treatment of IIH. Since this study[5] was performed using ip administration of *l*-alanine and *l*-glutamine the possibility to repeat this effect with oral administration must be considered. Therefore, in this investigation we compared the acute effect of oral administration of glucose, *l*-glutamine and *l*-alanine on glucose recovery. In addition, combined administrations of these amino acids (*l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine) were tested.

IIH rats that received oral administration of *l*-alanine showed higher glycemia ($P<0.05$) than IIH rats that received saline, glucose, *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine (Fig 2). Thus, it must be considered that our previous results[3-5] and the data showed here reinforce the suggestion that oral *l*-alanine could be better antidote than glucose to recovery IIH.

The worse result obtained with oral glucose (Fig 2) could be attributed to the possibility of a blunt in the counterregulatory mechanism of glucose recovery promoted by a transitory elevation of glycemia after glucose administration. In addition, the absence of glucose recovery with oral *l*-glutamine administration could be attributed to the fact that intestinal cells utilize this amino acid at high rates[9] reducing the amount disposable to the liver.

On the hand, an unexpected result was the absence of glucose recovery with the oral administration of *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine (Fig 2).

Since all amino acids orally administered in these experiments are partly metabolized in the intestine[9], it is hard to estimate exactly how much of the amount orally administered actually entering in the portal vein. To clarify this question and overcome limitations of *in vivo* experiments, we employed isolated liver from fasted rats, which received saturating concentration of gluconeogenic substrates and constant flux. This experimental approach discards the influence of hepatic glycogen catabolism, hepatic blood flux changes and the variability of blood gluconeogenic precursors[2-5].

Perfused livers from 24 h fasted rats produce negligible amounts of glucose in the absence of glucose precursors[16]

and the addition of gluconeogenic substrates increases the rate of glucose production in proportion to the amount of the glucose precursor until a saturating concentration was reached (results not shown). The values obtained were similar to livers from IIH and COG rats, except to *l*-alanine. For this amino acid the maximal hepatic glucose production from IIH was obtained with 5.0 $mmol.L^{-1}$, i.e., 50% of the concentration used to obtain the maximal hepatic glucose production in livers from COG rats, i.e., 10 $mmol.L^{-1}$ (Tab 1). Therefore, differently of *l*-glutamine and *l*-alanyl-l-glutamine, the ability of the liver to produce glucose from *l*-alanine during IIH was increased. In addition we observed that the catabolism of *l*-alanine in livers from IIH rats, inferred by the urea (Fig 4), *l*-lactate (Fig 5A) and pyruvate (Fig 5B) production, was favorable to *l*-lactate production in detriment of pyruvate production. This effect may be partly at least, as consequence of a increased $NADH/NAD^{+}$ ratio in the hypoglycemic rat liver.

Therefore, by using a saturating concentration of *l*-alanine, *l*-glutamine, *l*-glutamine + *l*-alanine or *l*-alanyl-l-glutamine we obtained data compatible with the view that the ability of the liver to produce glucose was increased or maintained during short term IIH. Part of these results can be explained by the fact that there is an increased release of

counterregulatory hormones during IIH, which antagonize the effects of insulin on the key enzymes of gluconeogenesis [17].

The lower hepatic glucose production from *l*-alanyl-l-glutamine exhibited by IIH and COG group was probably the result of the decreased catabolism of this dipeptide, inferred by the lower ($P<0.05$) urea (Fig 4), *l*-lactate (Fig 5A) and pyruvate (Fig 5B) production.

In summary, the data demonstrated that oral administration of *l*-alanine, but not *l*-glutamine, *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine affects glucose recovery. The favorable effect of *l*-alanine was partly at least the consequence of the higher ability of the livers from IIH rats to produce glucose from *l*-alanine if compared with *l*-glutamine, *l*-glutamine + *l*-alanine or *l*-alanyl-*l*-glutamine.

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LEGENDS

Tab 1. Maximal hepatic capacity to produce glucose (mmol.L^{-1}) from *l*-alanine, *l*-glutamine and *l*-alanyl-*l*-glutamine (*l*-ala-*l*-gln). The saturating concentration of each substrate were obtained by using increasing concentrations of these glucose precursors. The liver perfusion experiments were performed 30 min after insulin (IIH group) or saline (COG group) injection in 24 h-fasted rats.

Fig 1. Demonstrative experiments of glucose production from *l*-glutamine in perfused livers from 24 h-fasted rats that received insulin (IIH group) or saline (COG group). The effluent perfusate was sampled in 5 min intervals and analyzed for glucose. n=7. Mean±SD. AUC= Area Under Curve (μmol.g^{-1}). $^{b}P<0.05$ IIH vs COG group.

Fig 2. Effect of oral administration of saline, glucose (glu), *l*-glutamine (*l*-gln), *l*-alanine (*l*-ala), *l*-glutamine + *l*-alanine (*l*-gln + *l*-ala) and *l*-alanyl-*l*-glutamine (*l*-ala-*l*-gln) on glycemia of 24 h-fasted rats. All substances (100 mg.Kg^{-1}) were orally administrated 15 min after insulin (IIH group) or saline (COG group) injection. Glycemia

was measured 15 min, i.e., 30 min after insulin or saline injection. n=7. Mean±SD. [a]P>0.05 and [b]P<0.05 vs saline-COG, [d]P>0.05 and [e]P<0.05 vs saline-IIH.

Fig 3. Glucose production from saturating concentration of *l*-alanine (*l*-ala: 5 mmol.L^{-1} and 10 mmol.L^{-1} respectively to IIH and COG), *l*-glutamine (*l*-gln: 10 mmol.L^{-1} to IIH and COG), *l*-gln + *l*-ala: 10 mmol.L^{-1} + 5 mmol.L^{-1} to IIH and 10 mmol.L^{-1} + 10 mol.L^{-1} to COG) and *l*-alany-*l*-glutamine (*l*-ala-*l*-gln: 5 mmol.L^{-1} to IIH and COG) from livers of hypoglycemic (IIH group) and normoglycemic (COG group) 24 h-fasting rats. The livers were perfused as described in materials and methods. n=7. Means±SD. AUC=Area Under the Curve. [a]P>0.05 and [b]P<0.05 vs COG, [e]P<0.05 vs l-alanine-IIH.

Fig 4. Urea production from saturating concentration of *l*-alanine (*l*-ala: 5 mmol.L^{-1} and 10 mmol.L^{-1} respectively to IIH and COG), *l*-glutamine (*l*-gln: 10 mmol.L^{-1} to IIH and COG), *l*-gln + *l*-ala: 10 mmol.L^{-1} + 5 mmol.L^{-1} to IIH and 10 mmol.L^{-1} + 10 mol.L^{-1} to COG) and *l*-alany-*l*-glutamine (*l*-ala-*l*-gln: 5 mmol.L^{-1} to IIH and COG) from livers of hypoglycemic (IIH group) and normoglycemic (COG group) 24 h-fasted rats. The livers were perfused

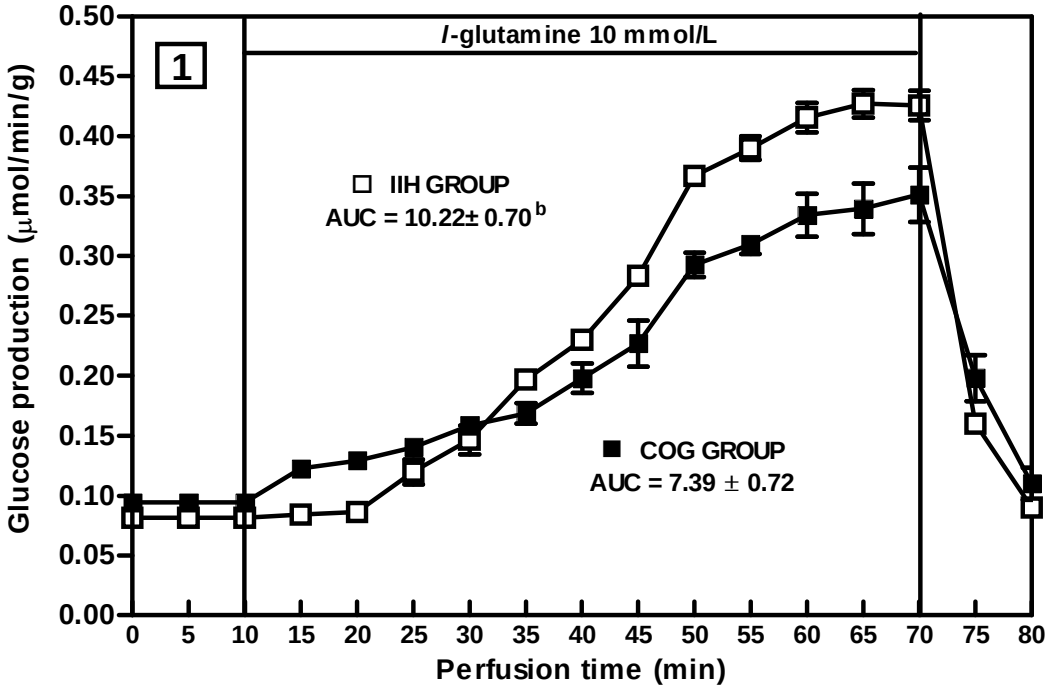
as described in materials and methods. *n*=7. Means±SD. AUC=Area Under the Curve. [b]*P*<0.05 vs COG.

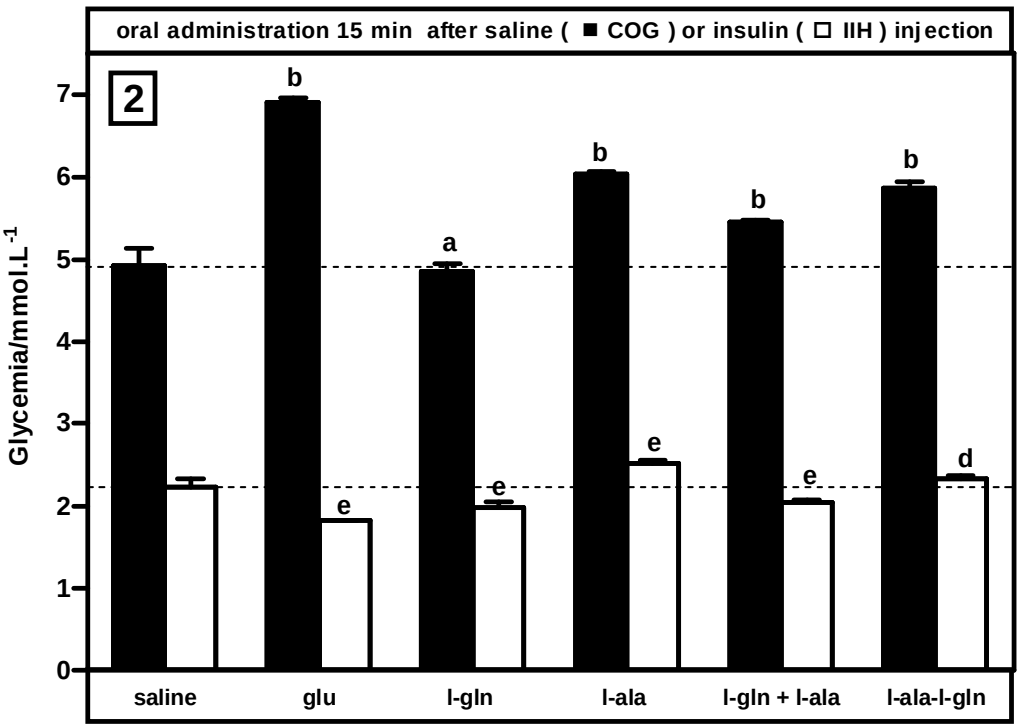
Fig 5. *l*-lactate (A) and pyruvate (B) production from saturating concentration of *l*-alanine (*l*-ala: 5 mmol.L^{-1} and 10 mmol.L^{-1} respectively to IIH and COG), *l*-gln + *l*-ala: (10 mmol.L^{-1} + 5 mmol.L^{-1} to IIH and 10 mmol.L^{-1} + 10 mol.L^{-1} to COG) and *l*-alany-*l*-glutamine (*l*-ala-*l*-gln: 5 mmol.L^{-1} to IIH and COG) from livers of hypoglycemic (IIH group) and normoglycemic (COG group) 24 h-fasted rats. The livers were perfused as described in materials and methods. *n*=7. Means±SD. AUC=Area Under the Curve. [a]*P*>0.05 and [b]*P*<0.05 vs *l*-lactate production COG; [d]*P*>0.05 and [e]*P*<0.05 vs pyruvate production COG.

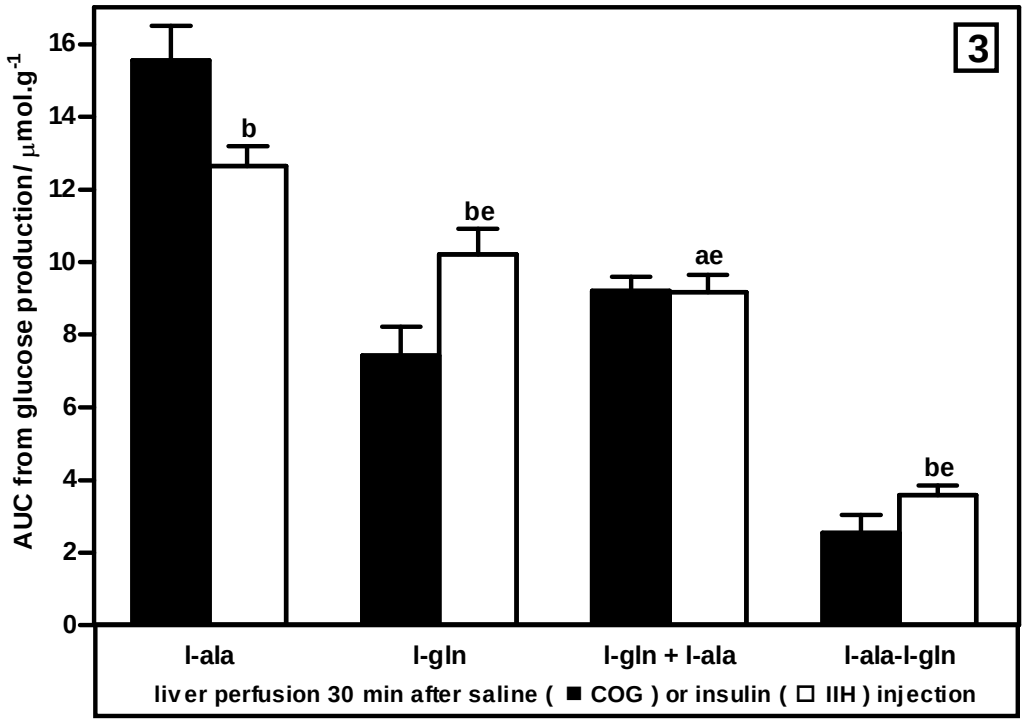
Tab 1.

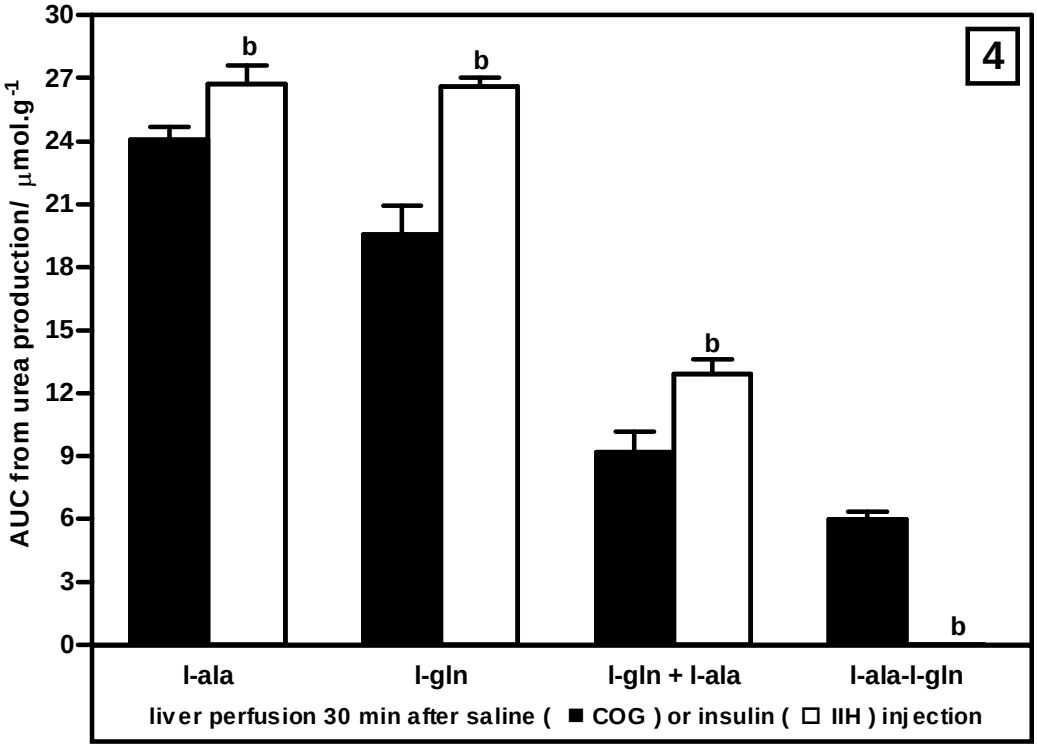
SATURATING CONCENTRATION (mM)		
Substrate	**COG group**	**IIH group**
***l*-alanine**	**10.0**	**5.0**
***l*-**	**10.0**	**10.0**
***l*-ala-*l*-gln**	**5.0**	**5.0**

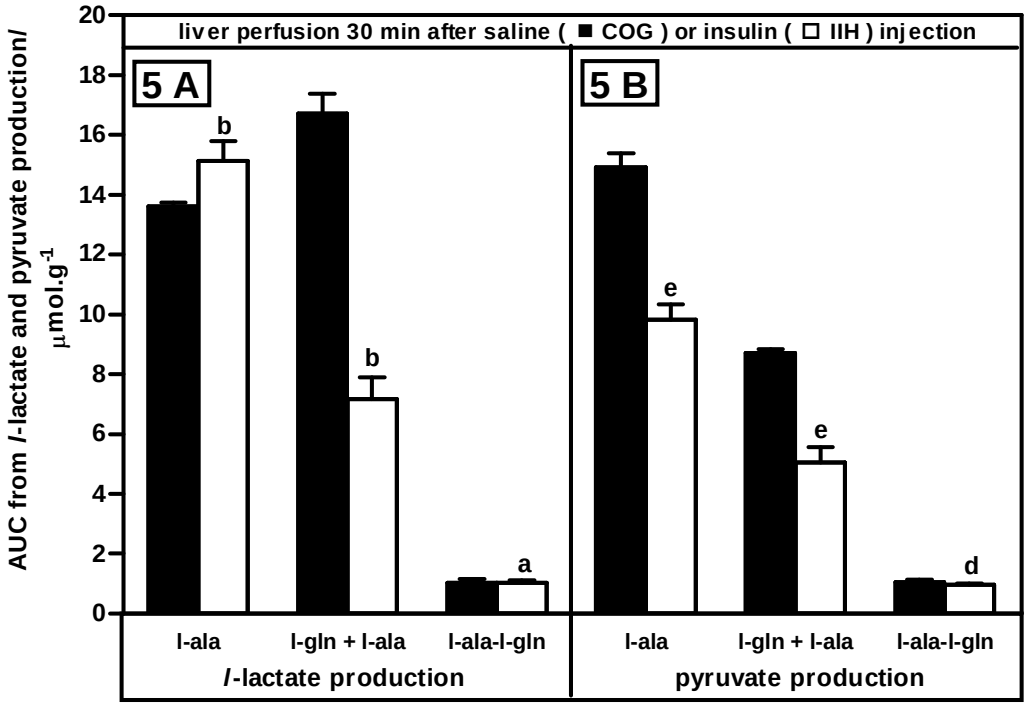
FIGURES


1
l-glutamine 10 mmol/L
IIH GROUP
AUC = 10.22± 0.70 b
COG GROUP
AUC = 7.39 ± 0.72
Glucose production (µmol/min/g)
Perfusion time (min)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80


oral administration 15 min after saline (■ COG) or insulin (□ IIH) injection
2
Glycemia/mmol.L-1
0
1
2
3
4
5
6
7
b
e
a
e
b
e
b
e
b
d
saline
glu
l-gln
l-ala
l-gln + l-ala
l-ala-l-gln


3
AUC from glucose production/ µmol.g-1
0
2
4
6
8
10
12
14
16
b
be
ae
be
l-ala
l-gln
l-gln + l-ala
l-ala-l-gln
liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection


4
AUC from urea production/ µmol.g-1
0
3
6
9
12
15
18
21
24
27
30
b
b
b
b
l-ala
l-gln
l-gln + l-ala
l-ala-l-gln
liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection


liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection
5 A
5 B
AUC from l-lactate and pyruvate production/ µmol.g-1
20
18
16
14
12
10
8
6
4
2
0
b
b
a
e
e
d
l-ala
l-gln + l-ala
l-ala-l-gln
l-ala
l-gln + l-ala
l-ala-l-gln
l-lactate production
pyruvate production

----------ARTIGO II

"Acute effects of isolated and combined L-alanine and L-glutamine on hepatic gluconeogenesis, ureagenesis and glycaemia recovery in experimental short-term insulin induced hypoglycaemia"

Acute effects of isolated and combined L-alanine and L-glutamine on hepatic gluconeogenesis, ureagenesis and glycaemia recovery in experimental short-term insulin induced hypoglycaemia

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Running Title: Effects of amino acids on gluconeogenesis and ureagenesis during hypoglycaemia

Key Words: hepatic gluconeogenesis, ureagenesis, hypoglycaemia, L-alanine, L-glutamine, insulin.

Abstract

The acute effects of isolated and combined L-alanine (L-Ala) and L-glutamine (L-Gln) on liver gluconeogenesis, ureagenesis and glycaemia recovery during short-term insulin induced hypoglycaemia (IIH) were investigated. For this purpose, 24-h fasted rats that received intraperitoneal injection regular insulin (1.0 U/Kg) or saline were compared. The studies were performed 30 min after insulin (IIH group) or saline (COG group) injection. Livers from IIH and COG groups were perfused with basal or saturating levels of L-Ala, L-Gln or L-Gln + L-Ala (L-G + L-A). The hepatic production of glucose, urea, L-lactate and pyruvate were markedly increased ($P < 0.001$) when the livers were perfused with saturating levels of L-Ala, L-Gln or L-G + L-A if compared with the basal levels of the same substrates. In addition, livers from IIH rats showed higher ability to produce glucose

and urea from saturating levels of L-Ala if compared with L-Gln or L-G + L-A. In agreement with the results obtained in isolated livers, the oral administration of L-Ala (100 mg/Kg) promoted better glycaemia recovery than L-Gln (100 mg/Kg) or the combination of L-G (50 mg/Kg) + L-A (50 mg/Kg). It can be concluded that L-Ala, but not L-Gln or L-G + L-A could help glucose recovery by a mechanism mediated, partly at least, due to the increased gluconeogenic and ureagenic efficiency.

Key Words: hepatic gluconeogenesis, ureagenesis, hypoglycaemia, L-alanine, L-glutamine, insulin.

Introduction

Glucose homeokinesis requires a continuous liver glucose production[1]. However, the insulin administration to treat diabetes could promote insulin induced hypoglycaemia (IIH) by decreasing the mobilisation of gluconeogenic substrates to the liver[2-4]. Thus, the possibility of the administration of hepatic glucose precursors to promote glucose recovery must be considered.

Therefore, since the first description of the treatment of hypoglycaemic symptoms with amino acids[5], studies in

experimental animals, healthy volunteers and patients with diabetes have shown the possibility to use new substances instead glucose to treat hypoglycemia. Among others, favorable results were obtained employing L-alanine[6], lipid[7], pyruvate[8], L-lactate[9] and glutamine dipeptide[10]. In agreement with these studies[5-10] we observed that the intraperitoneal administration of L-alanine or L-glutamine were more efficient than glucose itself to promote glycaemia recovery during IIH[11]. In addition, we found increased hepatic capacity to produce glucose from these amino acids[12]. However, it is necessary to emphasise that our previous studies[11,12] were done with 6 h-fasted rats where hepatic glycogenolysis contributed to the glucose production. In contrast, the present work used 24-h fasted rats, a favourable condition for gluconeogenesis, in which hepatic glycogen was completely depleted. Thus, the acute effect of isolated and combined infusion of the most important gluconeogenic amino acid, i.e., L-alanine[6] and the most abundant extracellular amino acid, i.e., L-glutamine[1] on gluconeogenesis was investigated. In addition, we expanded our previous studies[11,12] including a further investigation about the integration of gluconeogenesis/ureagenesis/ammoniagenesis to explain the higher liver glucose production and the better glucose recovery from oral L-Ala.

Material and methods

Animals

Male Wistar 24-h fasted rats weighing 180-220 g were employed. The manipulation followed the Brazilian law on the protection of animals. The rats were maintained under constant temperature (23°C) with automatically controlled photoperiod (12-h light/12-h dark).

Materials

Insulin (Novolin®) was purchased from Novo Nordisk (Brazil). L-Alanine (L-Ala) and L-Glutamine (L-Gln) were obtained from ICN Biochemichals (USA). All other reagents were of the highest purity obtainable.

Experimental short-term IIH

The first set of experiments was done to characterise a short-term IIH after an intraperitoneal injection of regular insulin (1.0 U/Kg). The values of glycaemia after insulin injection (IIH group) at 0, 15, 30 and 60 min were respectively 4.87, 3.26, 2.29 and 2.53 mmol/l. The values after saline injection (COG group) at 0, 15, 30 and 60 min were 4.87, 4.87, 4.84 and 4.84 mmol/l respectively. IIH was well-established 15 min after insulin

injection and so this period of time was selected for oral administration of the amino acids. In addition, considering that the lower glycaemia was observed 30 min after insulin injection this time was selected to measure the basal levels of blood glucose [13], L-Gln[14], L-Ala[15] and for the measurement of the hepatic gluconeogenesis and ureagenesis.

Liver perfusion technique

The rats were anaesthetised by an intraperitoneal injection of sodium pentobarbital (40 mg/Kg) and submitted to laparotomy. The livers were perfused *in situ* using Krebs Henseleit bicarbonate buffer (KHB), pH 7.4, saturated with O_2/CO_2 (95/5%). The perfusion fluid was pumped through a temperature controlled (37ºC) membrane oxygenator prior to entering the liver via portal vein.

The liver perfusion experiments were executed according to the protocol illustrated in Fig. 1. After a pre-perfusion period (10 min), saturating concentration of L-Ala was dissolved in the perfusion fluid and infused between the 10^{th} and 70^{th} 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 the concentration of glucose, pyruvate[17], L-lactate[16], urea[18] and ammonia[18] were determined. The differences in the glucose or pyruvate, L-lactate, urea and ammonia production during (10-70 min) and before (0-10 min) the infusion of L-Ala allowed calculate the areas under the curves (AUC), expressed as μmol/g. Similar procedure was done when basal and saturating concentrations of L-Ala, L-Gln or L-Gln + L-Ala (L-G + L-A) were used as the gluconeogenic substrates. Thus, the AUC showed in Figures 2-7 were obtained from similar experiments as that illustrated in Fig. 1.

Determination of the liver capacity to produce glucose from L-Gln and L-Ala

Liver perfusion experiments using increasing concentrations of L-Gln or L-Ala were performed. The addition of the amino acids increased the rate of glucose production in a dose dependent way proportionately to the amount of precursor until the liver maximal capacity was reached, i.e., the lower concentration in which the maximal hepatic glucose production was obtained (Fig. 2).

Effect of oral administration of amino acids on glycaemia recovery

L-Gln (100 mg/Kg), L-Ala (100 mg/Kg) and L-G (50 mg/Kg) + L-A (50 mg/Kg) were orally (gavage) administered 15 min after insulin injection. Moreover, two additional subgroups that received oral glucose (100 mg/Kg) or saline 15 min after insulin (IIH group) injection were included. Blood samples were collected by decapitation and glycaemia was determined 15-min later, i.e. 30 min after insulin injection (Fig. 8).

Statistical analysis

Results are reported as means ± S.D. The program GraphPad Prism (version 6.0) was used to calculate the AUC. Data were analysed statistically by the unpaired Student's *t*-test.

Results

IIH decreased ($P < 0.001$) the blood levels of glucose and L-Gln but that of L-Ala remained unchanged (Table 1).

The liver capacity to produce glucose from increasing concentrations of L-Ala and L-Gln in livers from IIH and COG rats were evaluated. The maximal glucose production, which reflects the liver capacity to produce glucose from a

saturating concentration of L-Ala were 10.0 mM (COG group) and 5.0 mM (IIH group) whereas for L-Gln the value was 10.0 mM for both groups (Fig. 2).

The basal values described in Table 1 and the saturating values illustrated by Fig 2 were used to design the experiments showed in figures 3-7.

The liver production of glucose (Fig. 3), urea (Fig. 4), L-lactate (Fig. 6) and pyruvate (Fig. 7) was increased ($P<0.001$) by several folds in the presence of saturating levels of L-Ala, L-Gln or L-G + L-A if compared with the basal values of the same amino acids.

In livers from IIH rats the capacity to produce glucose (Fig. 3B) from saturating levels of L-Gln was higher ($p<0.001$) than that of the COG group. On the other hand, the maximal glucose production from L-Ala in livers from IIH rats was about 20% lower than that of the COG group (Fig. 3B). In addition, capacity to produce glucose (Fig. 3B) from saturating levels of L-G + L-A was not different (IIH vs. COG group).

Livers from IIH rats also showed higher capacity to produce urea (Fig. 4B) from saturating levels of L-Ala, L-Gln or L-G + L-A (IIH group vs. COG group). However, differently of L-Gln or L-G + L-A, ammonia production from saturating levels of L-Ala was negligible (Fig. 5B).

Finally, the oral administration of L-Ala promoted glucose recovery, but this effect was not observed if L-Gln or L-G + L-A were used instead L-Ala (Fig 8).

Discussion

In contrast to carbohydrates and lipids the liver catabolism of amino acids generate ammonia which must be disposed of[1]. Therefore, if the glucose precursor is an amino acid, hepatic gluconeogenesis and ureagenesis, that share common intermediates, must be activated simultaneously[19]. In agreement with this point of view the liver production of glucose (Fig. 3) and urea (Fig. 4) increased ($P<0.001$) several folds during the infusion of saturating levels of L-Ala, L-Gln or L-G + L-A if compared with the infusion of the basal concentration of these amino acids.

However, the hepatic gluconeogenesis (Fig. 3) and ureagenesis (Fig. 4) performance from L-Ala, L-Gln and L-G + L-A were different. At the moment, it is important to emphasise the main aspects of ammonia production. Thus, differently of Gln or L-G + L-A (Fig. 5), saturating levels of L-Ala produced a negligible amount of ammonia. A possible explanation for this result is the fact that the ammonia released from L-Ala catabolism is 50% than that obtained with equimolar amount of L-Gln[19]. In addition, the maximal

urea production from L-Ala in livers from IIH rats was higher than that of the COG group. In addition, since the maximal urea production from L-Ala was obtained with 50% of the concentration used to obtain the same effect in livers from COG group (Fig 2), we can conclude that the liver ability to produce urea from saturating levels of L-Ala was increased in the IIH group.

Since the rate of gluconeogenesis from L-Gln is lower than L-Ala (Fig. 3) and considering that the transformation of L-Gln to glucose does not involve the step inhibited by ammonia, i.e., pyruvate carboxylation[21], other mechanism must be involved in this difference. In principle, a possibility can be raised from the fact that L-Gln increases the intramitochondrial concentration of ammonia, which decreased the intermediates of TCA cycle leading to depletion of ATP[20]. In addition it must be considered that requirement of ATP to maintain gluconeogenesis/ureogenesis from L-Gln is higher than L-Ala. Therefore, the absence of additive effect of L-Gln on hepatic glucose production from L-Ala (Fig. 3) could be consequence of the intensification of ammonia production when these amino acids were infused with L-Ala (Fig. 5). Consequently, we can suggest that the better performance of L-Ala to produce glucose if compared with L-Gln or L-G + L-A

was partly at least consequence of the lower amount of ammonia produced during its catabolism.

The liver capacity to produce glucose (Fig. 3) from saturating levels of L-Gln was increased ($p<0.001$) in IIH rats (IIH vs. COG group). This result was the consequence of the increased catabolism of these amino acids as inferred by the higher ($p<0.001$) urea (Fig. 4) production, which support the generation of gluconeogenic intermediaries[22]. Differently of L-Gln, the maximal glucose production from L-Ala in livers from IIH rats was about 20% lower than that of the COG group. However, it has to be considered that the maximal glucose production from L-Ala was obtained with 50% of the concentration used to obtain the same effect in livers from COG group (Fig 2). Thus, the liver ability to produce glucose from saturating levels of L-Ala was increased in the IIH group. In agreement with this suggestion, livers from IIH rats showed a favorable cytosolic potential redox for gluconeogenesis, i.e., an increased $NADH/NAD^+$ ratio, inferred by the higher ($p<0.001$) L-lactate (Fig 6) and lower ($p<0.001$) pyruvate (Fig 7) production.

The mechanism involved in the increased ability and capacity to produce glucose under short term IIH also involves the increased release of counterregulatory hormones[23, 24] which stimulates the uptake and catabolism of

amino acids and activates the enzymes alanine aminotraneferase, glutaminase, phosphoenol-pyruvate carboxykinase and glucose-6-phosphatase[25-28].

Since the blood levels of L-Ala and L-Gln were respectively maintained and decreased (Tab. 1) and isolated livers from IIH rats showed higher ability and capacity to produce glucose from saturating levels of L-Ala and L-Gln respectively (Fig. 3) we decided to verify if isolated or combined L-Ala and L-Gln orally administered could help glycaemia recovery. As showed by figure 8 the best glycaemia recovery obtained with L-Ala, could be attributed not only to its increased portal disposability[1] but also an intensification of the glucagon secretion[27]. In addition, the worse result, obtained with oral glucose (Fig 8), could be imputed to the possibility of a blunt in the release of counterregulatory hormones promoted by a transitory elevation of portal glycaemia after its oral administration. On the other hand, the absence of glycaemia recovery with oral L-Gln could be ascribed to the fact that the intestinal cells utilize this amino acid at high rates[1] reducing the amount disposable to the liver. On the other hand, the lower ($P<0.001$) glycaemia of IIH rats that received L-G + L-A, was partly at least the result of the decreased catabolism of both amino acids, when infused simultaneously at saturating levels, as inferred by the

lower hepatic glucose (Fig. 3B), urea (Fig. 4B), L-lactate (Fig. 5B) and pyruvate (Fig. 6B) production (L-Gln or L-Ala vs. L-G + L-A).

Finally, our previous results[11,12] and the data showed here reinforce the suggestion that oral L-Ala could be better than glucose to promote glycaemia recovery. However, considering the possibility of the inhibition of the liver glucose production with high concentration of L-Ala (Fig. 2), the applicability of these results, with particular focus on their superiority on glucose administration await further experimental and clinical studies.

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LEGENDS

Fig. 1. Demonstrative experiment of glucose, urea, ammonia, pyruvate and L-lactate production from saturating concentration of L-alanine in perfused livers from 24-h fasted rats. The effluent perfusate was sampled in 5 min intervals and determined for glucose, urea, ammonia, pyruvate and L-lactate. The areas under the curves (AUC), expressed as µmol/g, were obtained from the difference between the values during and before L-alanine infusion.

Fig. 2. Glucose production from increasing concentrations of L-Alanine **(2A)** and L-Glutamine **(2B)** in livers from hypoglycaemic (IIH group) and normoglycaemic (COG group) 24 h-fasted rats. The areas under the curves (AUC) were obtained as described in materials and methods. The AUC values were expressed as means ± S.D of 7 liver perfusion experiments. $^{*}P < .001$ vs. 10 mM L-alanine COG, $^{**}P < .001$ vs 10 mM L-alanine IIH, $^{***}P < .001$ vs 10 mM L-glutamine COG and $^{****}P < .001$ vs. 10 mM L-glutamine IIH.

Fig. 3. Glucose production from basal **(3A)** and saturating **(3B)** concentrations of L-Alanine, (L-Ala), L-Glutamine (L-Gln), L-Gln + L-Ala (L-G + L-A) in livers from hypoglycaemic

(IIH group) and normoglycaemic (COG group) 24 h-fasted rats. Basal (Table 1) and saturating values (Fig. 2) of L-Gln and L-Ala were previously described. The areas under the curves (AUC), obtained as described in materials and methods, were expressed as means ± S.D of 7 liver perfusion experiments. $^{*}P <$ .0001 vs COG and $^{**}P <$ .0001 vs saturating.

Fig. 4. Urea production from basal (**4A**) and saturating (**4B**) concentrations of L-Alanine, (L-Ala), L-Glutamine (L-Gln), L-Gln + L-Ala (L-G + L-A) in livers from hypoglycaemic (IIH group) and normoglycaemic (COG group) 24 h-fasted rats. Basal (Table 1) and saturating values (Fig. 2) of L-Gln and L-Ala were previously described. The areas under the curves (AUC), obtained as described in materials and methods, were expressed as means ± S.D of 7 liver perfusion experiments. $^{*}P <$ .0001 vs COG and $^{**}P <$ .0001 vs. saturating.

Fig. 5. Ammonia production from basal (**5A**) and saturating (**5B**) concentrations of L-Alanine, (L-Ala), L-Glutamine (L-Gln), L-Ala + L-Ala (L-G + L-A) in livers from hypoglycaemic (IIH group) and normoglycaemic (COG group) 24 h-fasted rats. Basal (Table 1) and saturating values (Fig. 2) of L-Gln and L-Ala were previously described. The areas under the

curves (AUC), obtained as described in materials and methods, were expressed as means ± S.D of 7 liver perfusion experiments. $^{*}P <$ .0001 vs. COG and $^{**}P <$ .0001 vs. saturating.

Fig. 6. L-lactate production from basal (**6A**) and saturating (**6B**) concentrations of L-Alanine, (L-Ala) or L-Gln + L-Ala (L-G + L-A) in livers from hypoglycaemic (IIH group) and normoglycaemic (COG group) 24 h-fasted rats. Basal (Table 1) and saturating values (Fig. 2) of L-Gln and L-Ala were previously described. The areas under the curves (AUC), obtained as described in materials and methods, were expressed as means ± S.D of 7 liver perfusion experiments. $^{*}P <$ .0001 vs. COG and $^{**}P <$ .0001 vs. saturating.

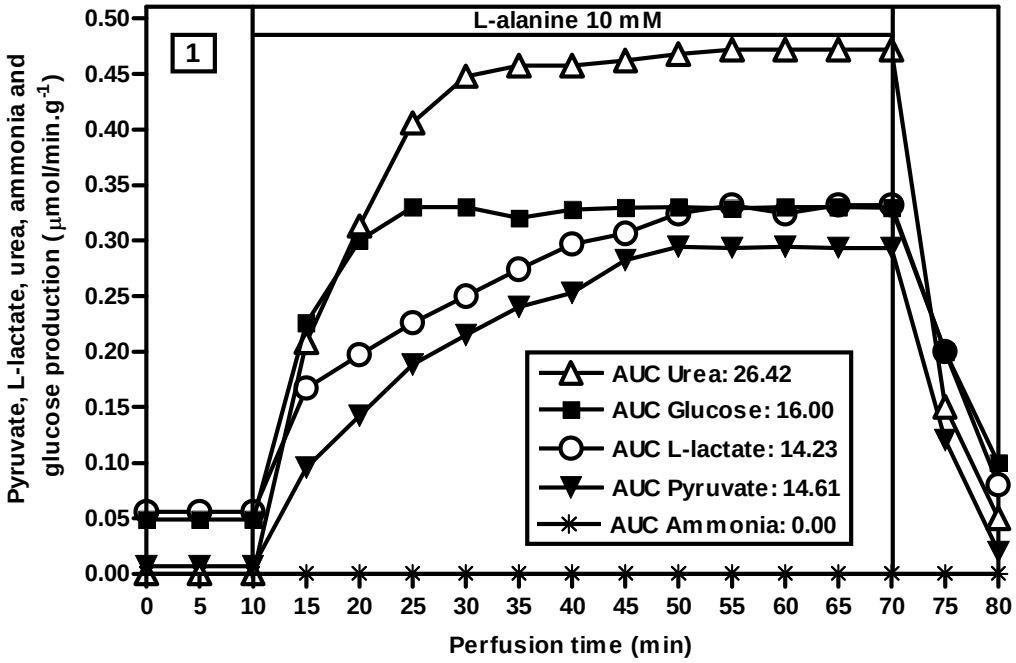
Fig. 7. Pyruvate production from basal **(7A)** and saturating **(7B)** concentrations of L-Alanine (L-Ala) or L-Gln + L-Ala (L-G + L-A) in livers from hypoglycaemic (IIH group) and normoglycaemic (COG group) 24 h-fasted rats. Basal (Table 1) and saturating values (Fig. 2) of L-Gln and L-Ala were previously described. The areas under the curves (AUC), obtained as described in materials and methods, were expressed as means ± S.D of 7 liver perfusion experiments. $^{*}P <$ .0001 vs. COG and $^{**}P <$ .0001 vs. saturating.

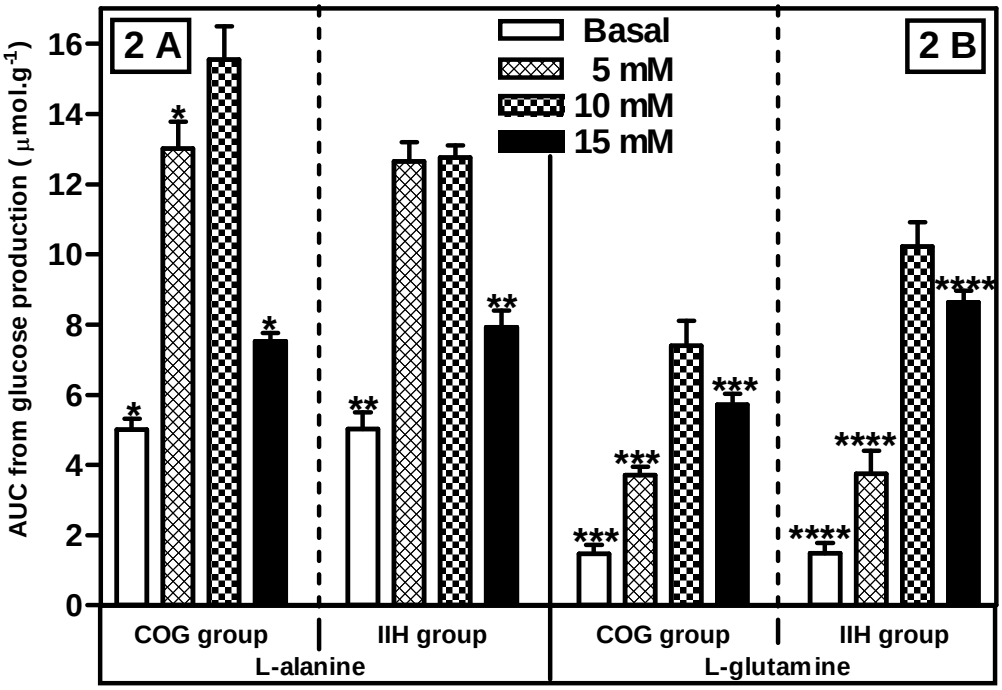
Fig. 8. Effect of oral administration of Saline, Glucose (Glu), L-Glutamine (L-Gln), L-Alanine (L-Ala) or L-Glutamine + L-Alanine (L-G + L-A) on glycaemia of 24 h-fasted rats. All substances (100 mg/Kg) were orally (gavage) administered 15 min after insulin injection. Glycaemia was measured from blood samples collected 30 min after insulin injection. The values were expressed as means ± S.D of 7 experiments. $^{*}P < .0001$ vs. saline.

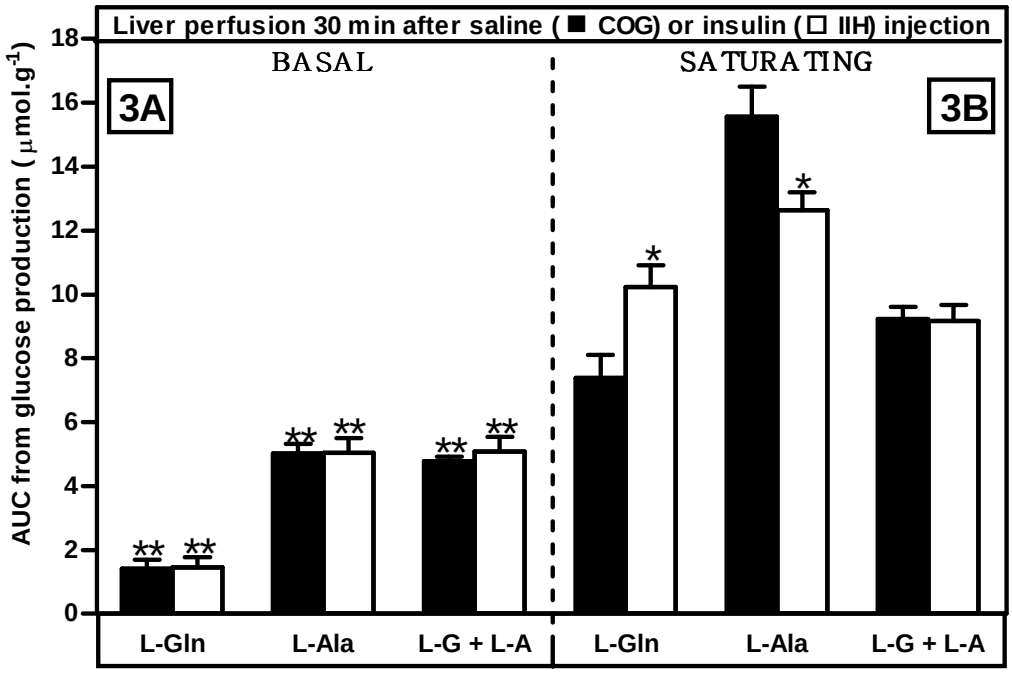
Table 1

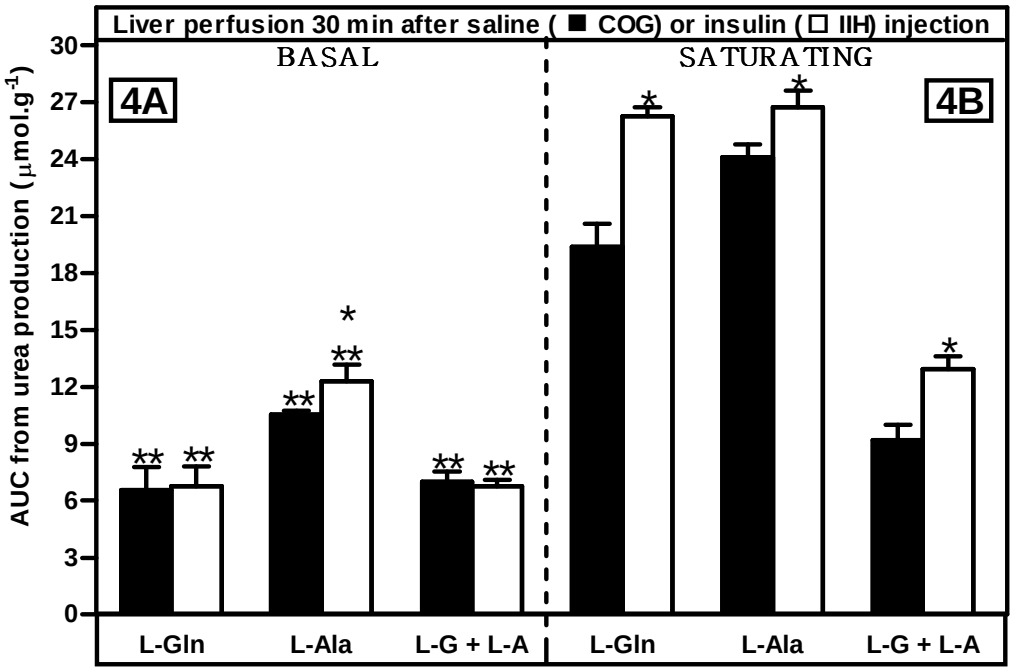
Blood levels (mmol/l) of glucose, L-Alanine (L-Ala) and L-Glutamine (L-Gln) 30 min after intraperitoneal saline (COG group) or insulin (IIH group) administration in 24-h fasted rats. The results were expressed as means ± S.D for the indicated number (n) of animals.

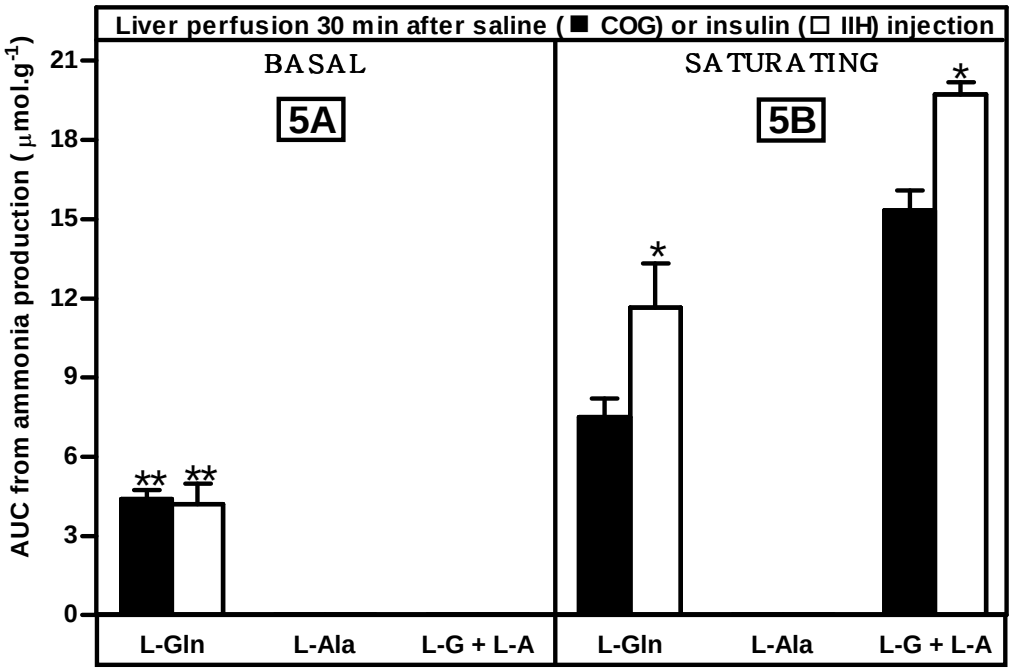
Basal	COG group (n: 6-8)	IIH group (n: 6-8)	*P*
Glucose	4.84 ± 0.28	2.29 ± 0.18	<
L-Ala	0.62 ± 0.02	0.61 ± 0.07	
L-Gln	2.00 ± 0.21	1.41 ± 0.01	<

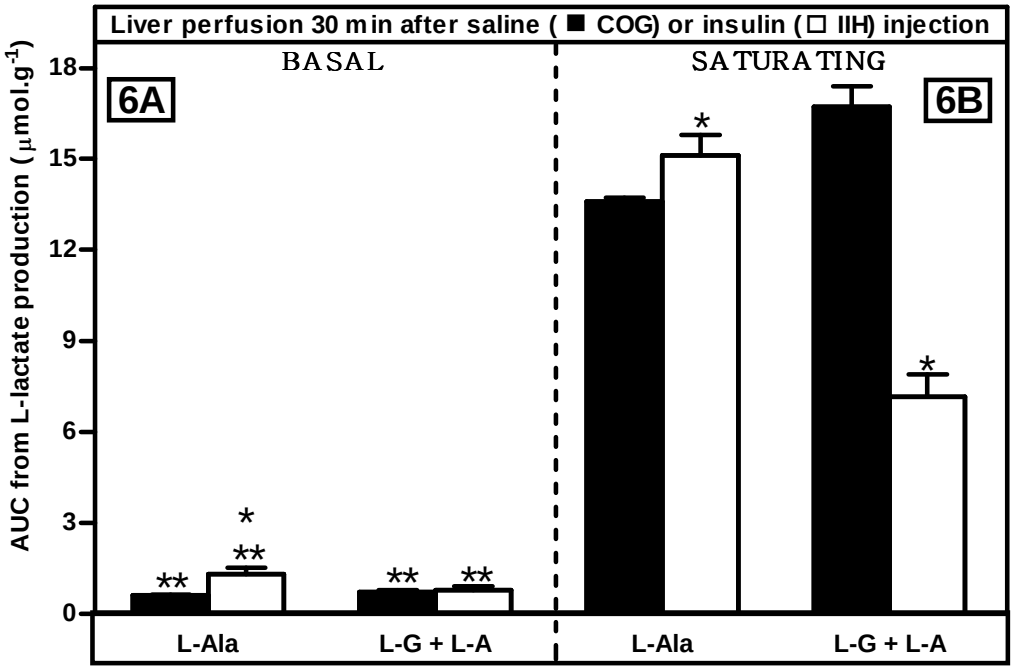

1
L-alanine 10 mM
Pyruvate, L-lactate, urea, ammonia and glucose production (µmol/min.g-1)
Perfusion time (min)
AUC Urea: 26.42
AUC Glucose: 16.00
AUC L-lactate: 14.23
AUC Pyruvate: 14.61
AUC Ammonia: 0.00

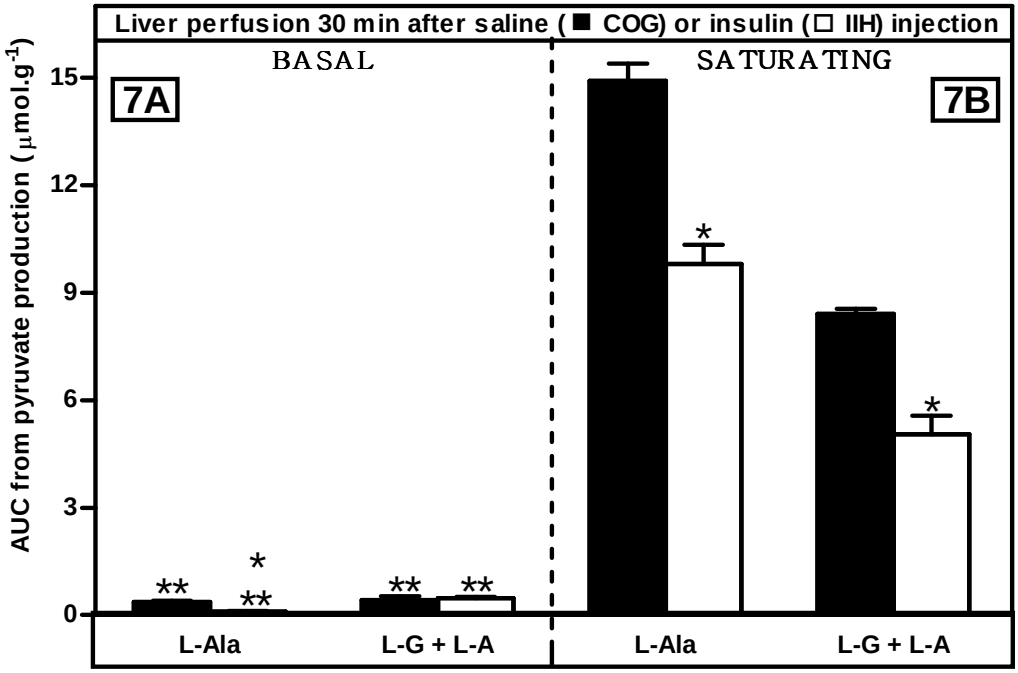

2 A
2 B
Basal
5 mM
10 mM
15 mM
AUC from glucose production (µmol.g-1)
COG group
IIH group
COG group
IIH group
L-alanine
L-glutamine

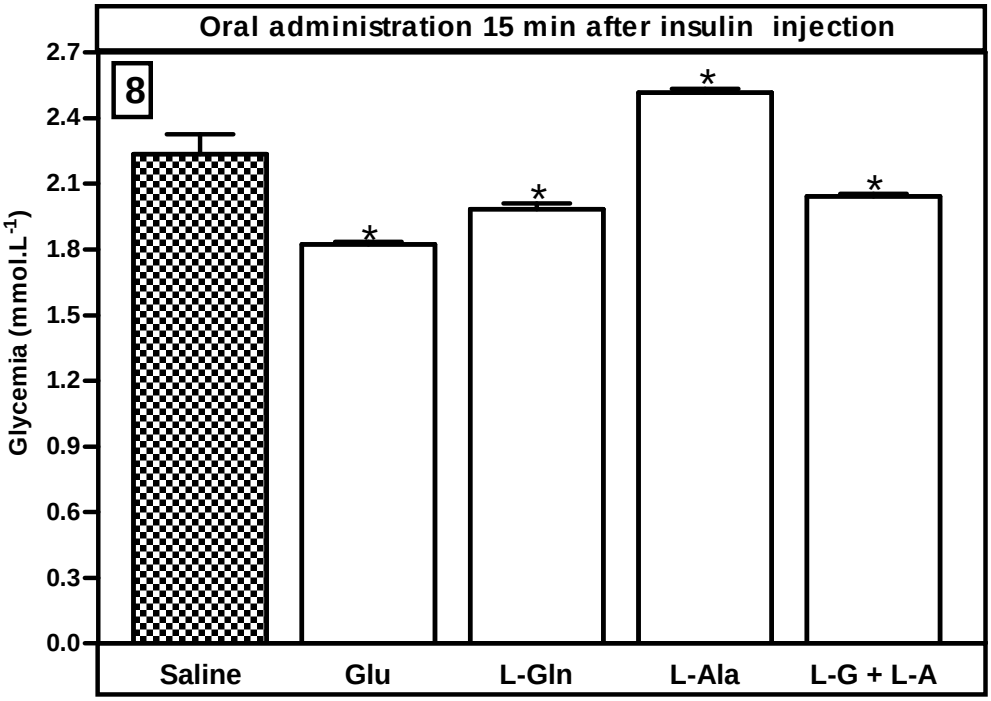

Liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection
BASAL
SATURATING
3A
3B
AUC from glucose production (µmol.g-1)
0
2
4
6
8
10
12
14
16
18
L-Gln
L-Ala
L-G + L-A
L-Gln
L-Ala
L-G + L-A


Liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection
BASAL
SATURATING
4A
4B
AUC from urea production (µmol.g-1)
0
3
6
9
12
15
18
21
24
27
30
L-Gln
L-Ala
L-G + L-A
L-Gln
L-Ala
L-G + L-A


Liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection
AUC from ammonia production (µmol.g-1)
BASAL
SATURATING
5A
5B
0
3
6
9
12
15
18
21
L-Gln
L-Ala
L-G + L-A
L-Gln
L-Ala
L-G + L-A


Liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection
AUC from L-lactate production (µmol.g-1)
BASAL
SATURATING
6A
6B
0
3
6
9
12
15
18
L-Ala
L-G + L-A
L-Ala
L-G + L-A


Liver perfusion 30 min after saline (■ COG) or insulin (□ IIH) injection
BASAL
SATURATING
7A
7B
AUC from pyruvate production (µmol.g-1)
15
12
9
6
3
0
**
*
**
**
**
*
*
L-Ala
L-G + L-A
L-Ala
L-G + L-A


Oral administration 15 min after insulin injection
8
Glycemia (mmol.L-1)
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
*
*
*
*
Saline
Glu
L-Gln
L-Ala
L-G + L-A

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