SANDONAID ANDREI GEISLER

INVESTIGAÇÃO DA CAPACIDADE GLICONEOGÊNICA EM FÍGADO DE RATOS SUBMETIDOS À HIPOGLICEMIA INDUZIDA POR GLIBENCLAMIDA

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

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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.

Orientador: Prof. Dr. Roberto Barbosa Bazotte

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BIOGRAFIA

Sandonaid Andrei Geisler nasceu em Rio dos Cedros/SC em 21/03/1978. Possui Graduação em Ciências Biológicas (Licenciatura) pela Universidade Estadual do Oeste do Paraná – Extensão Santa Helena, onde realizou estágio e pesquisas em Análise do comportamento de ratos Wistar submetidos à ingesta de água com glifosato e organoclorados. É pós-graduando do Programa de Pós-Graduação em Ciências Biológicas (área de concentração - Biologia Celular e Molecular) pela Universidade Estadual de Maringá, onde realizou pesquisas que investigaram a capacidade gliconeogênica de fígado de ratos submetidos à hipoglicemia induzida por glibenclamida.

Canção Mínima

No mistério do sem-fim
equilibra-se um planeta

E, no planeta, um jardim,
e, no jardim, um canteiro;
no canteiro uma violeta,
e, sobre ela, o dia inteiro,

entre o planeta e o sem-fim
a asa de uma borboleta

Cecília Meireles

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À Deus por ter guiado meu caminho.

Ao meu maior patrimônio, minha família, pois sem eles eu não teria alcançado esta vitória.

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A todos, o meu muito obrigado!!!

APRESENTAÇÃO

Esta dissertação de Mestrado foi redigida na forma de um artigo científico **“Investigation of the gluconeogenic capacity in livers from rats submitted to glibenclamide induced hypoglycemia”** em consonância com as normas do Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá e da revista ***Biological & Pharmaceutical Bulletin*** (ISSN: 0918-6158).

Este estudo representa a continuidade dos trabalhos desenvolvidos pelo grupo de pesquisa do Laboratório de Farmacologia Endócrina que investiga os mecanismos de manutenção, prevenção e recuperação da glicemia durante a hipoglicemia. Neste sentido, o presente trabalho investigou a capacidade neoglicogênica hepática de ratos submetidos à hipoglicemia induzida por glibenclamida.

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RESUMO GERAL

INTRODUÇÃO - Apesar do fato de que concentrações fisiológicas de insulina inibem a gliconeogênese hepática, concentrações supra fisiológicas que ocorrem durante a insulinoterapia, estimulam a gliconeogênese. Este efeito paradoxal pode ser explicado pela liberação dos hormônios contra-reguladores durante a hipoglicemia induzida por altas doses insulina, os quais sobrepõem o efeito inibitório da insulina sobre a gliconeogênese hepática. Assim como a injeção de insulina, as sulfoniluréias também promovem hipoglicemia, efeito este atribuído à liberação de insulina. Deste modo, um efeito esperado da hipoglicemia induzida por sulfoniluréia (HIS) seria uma ativação da gliconeogênese. Contudo, sulfoniluréias infundidas diretamente no fígado inibem a gliconeogênese, efeito este mediado, pelo menos parcialmente, pela inativação da função mitocondrial. **OBJETIVO -** Portanto, considerando estas influências opostas na gliconeogênese hepática, decidimos investigar se a gliconeogênese se encontra ativada ou inibida em fígados de ratos submetidos a uma HIS. Considerando que ratos não diabéticos constituem um bom modelo experimental para se investigar uma HIS e considerando que fígados de ratos perfundidos *in situ*, refletem as condições *in vivo* do animal imediatamente antes do isolamento do fígado, os experimentos foram realizados utilizando esta abordagem experimental. Para alcançar este objetivo, empregou-se glibenclamida, uma sulfoniluréia utilizada no tratamento do diabetes mellitus tipo 2. **MATERIAIS E MÉTODOS -** Ratos Wistar machos (180-220 g) em jejum de 24 h foram utilizados. Os procedimentos experimentais foram aprovados pelo comitê de ética da Universidade Estadual de Maringá, PR, Brasil. Inicialmente, um experimento preliminar para caracterizar a HIS após administração oral (intragástrica) de glibenclamida (10 mg/kg) foi realizado. O sangue foi obtido por decapitação. Os valores obtidos para a glicemia 30, 60, 90, 120 e 150 min (média ± D.P, n = 6) após administração oral de glibenlamida foram 111.9 ± 13.68, 75.33 ± 4.5, 63.12 ± 3.04, 60.5 ± 2.74 e 71.96 ± 3.84 mg/dl, respectivamente. Assim, para verificar o efeito da HIS na gliconeogênese hepática, os experimentos foram feitos 2 h após a administração de glibenclamida (grupo HIS), quando os menores valores de glicemia foram alcançados. O grupo controle normoglicêmico (grupo controle) foi representado por animais que receberam salina via oral. Para os experimentos de perfusão de fígado os ratos foram anestesiados com tiopental sódico (45 mg/kg) e em seguida submetidos à laparotomia. Os fígados foram perfundidos *in situ* com tampão Krebs-Henseileit Bicarbonato (KHB). Após um período de pré-infusão (10 min com KHB), os substratos gliconeogênicos (L-alanina, L-glutamina, L-lactato, piruvato ou glicerol) foram

dissolvidos no KHB, seguido por um período de pós-infusão (10 min com apenas KHB) para permitir o retorno aos valores basais. Amostras do líquido efluente da perfusão foram coletadas em intervalos de 5 minutos e a produção hepática de glicose e uréia foram analisadas. As diferenças na produção de glicose e uréia durante e antes da infusão de substratos gliconeogênicos permitiram calcular a área sob a curva (AUC). A adição de L-alanina, piruvato, L-glutamina ou glicerol aumentam proporcionalmente a taxa da produção de glicose até os valores saturantes serem alcançados. Assim, usando uma concentração saturante de precursores hepáticos de glicose, é possível medir a capacidade máxima de produção hepática a partir de cada substrato gliconeogênico. Assim, a capacidade gliconeogênica no fígado de ratos que receberam glibenclamida oral (grupo HIS) ou salina (grupo controle) foram comparadas utilizando concentração saturante de L-alanina (5 mM), L-glutamina (5 mM), L-lactato (2 mM), piruvato (5 mM) ou glicerol (2 mM). **RESULTADOS -** Na primeira série de experimentos, fígados de ratos que receberam solução salina via oral (grupo controle) ou glibenclamida por via oral (grupo HIS) foram infundidos com L-alanina (5 mM). Fígados de ratos HIS apresentaram menor ($p < 0,05$) produção de glicose e maior ($p < 0,05$) produção de piruvato em comparação aos fígados de ratos controle. No entanto, a produção de uréia e de L-lactato foi similar nos dois grupos. Na segunda série de experimentos os fígados de ratos controle e HIS foram infundidos com piruvato (5 mM). Fígados de ratos HIS apresentaram menor ($p < 0,05$) produção de glicose e maior ($p < 0,05$) produção L-lactato em comparação aos ratos controle. No terceiro conjunto de experimentos fígados de ratos controle e HIS foram perfundidos com L-glutamina (5 mM). Fígados de ratos HIS apresentaram menor ($p < 0,05$) produção de glicose em comparação a fígados de ratos controle. No entanto, a produção de uréia foi semelhante. Na quarta série de experimentos os fígados de ratos controle e HIS foram infundidos com L-lactato (2 mM), ou glicerol (2 mM). Fígados de ratos HIS apresentaram menor ($p < 0,05$) produção de glicose a partir de L-lactato (2mM). Todavia, a produção de glicose apartir do Glicerol (2 mM) foi semelhante nos dois grupos. Na quinta série de experimentos, fígados de ratos HIS foram infundidos com L-alanina em concentração fisiológica (0,45 mM) ou saturante (5 mM). Fígados infundidos em condições saturantes de L-alanina apresentaram maior ($p < 0,05$) produção de glicose e uréia em comparação aos fígados infundidos com L-alanina em concentração fisiológica. Na sexta série de experimentos os fígados de ratos HIS foram infundidos com L-glutamina em concentração fisiológica (2 mM) e saturante (5 mM). Fígados infundidos com L-glutamina em concentração saturante apresentaram maior ($p < 0,05$) produção de glicose e

uréia do que os fígados infundidos em concentração fisiológica de L-glutamina. **DISCUSSÃO -** O primeiro substrato gliconeogênico investigado foi a L-alanina, que atravessa a membrana celular hepática para ser convertida em piruvato, que por sua vez a partir do citosol entra na mitocôndria e é carboxilado e deixa a mitocôndria como aspartato ou malato. No citosol o malato ou o aspartato é convertido em oxaloacetato e, em seguida, a fosfoenolpiruvato e após várias etapas será convertido a glicose e liberado dos hepatócitos. Uma vez que este complexo percurso depende do abastecimento de oxigênio e vários compartimentos celulares, a produção de glicose, L-lactato e piruvato a partir da L-alanina constituem um marcador da integridade do hepatócito. Desta forma, utilizando uma concentração saturante de L-alanina, demonstrou-se que a capacidade de produção de glicose em fígados de ratos submetidos à HIS foi diminuída ($p < 0,05$). Este resultado não poderia ser atribuído ao diminuído catabolismo da L-alanina, uma vez que a produção de uréia a partir deste aminoácido foi inalterada. Além disso, considerando que a produção de piruvato a partir da L-alanina em fígados de ratos HIS se elevou ($p < 0,05$), a possibilidade de uma menor entrada de piruvato na via gliconeogênica deve ser considerada. Em concordância com esta proposição, fígados de ratos submetidos à HIS apresentaram redução ($p < 0,05$) de produção de glicose, não apenas a partir de piruvato, mas também a partir de L-lactato. Além disso, a produção hepática de glicose a partir da L-glutamina, que entra na via gliconeogênica após a piruvato carboxilase (PC), também foi diminuída ($p < 0,05$). Este resultado, não pode ser atribuído à diminuição do catabolismo da L-glutamina, pois a produção de uréia a partir deste aminoácido foi inalterada. No entanto, a produção de glicose a partir de glicerol, cuja entrada na gliconeogênese ocorre em uma etapa pós mitocondrial permaneceu inalterada. No seu conjunto, os resultados sugerem que a inibição da gliconeogênese promovida pela glibenclamida supera a intensificação da gliconeogênese hepática em resposta à hipoglicemia. Além disso, se este efeito da glibenclamida ocorrer *in vivo*, o resultado seria uma intensificação da eficácia desta droga como agente hipoglicemiante. No entanto, apesar da diminuição da capacidade hepática de produção de glicose durante uma HIS, a possibilidade de administrar substratos gliconeogênicos durante esta condição deve ser considerada. Esta afirmação foi baseada no fato de que, durante a HIS, a produção hepática de glicose a partir de L-alanina e L-glutamina foi menor. Porém, mantida e influenciada pela disponibilidade destes aminoácidos. Finalmente, nossos resultados e as considerações aqui apresentadas, abrem a possibilidade da administração de substratos gliconeogênicos no tratamento da HIS, particularmente quando a terapia com glicose não é eficaz.

GENERAL ABSTRACT

INTRODUCTION - In spite of the fact that physiological levels of insulin inhibit liver gluconeogenesis, supraphysiological levels of insulin which occur during insulin therapy stimulates gluconeogenesis. This paradoxical effect can be explained by the fact that the counterregulatory hormones released during hypoglycemia induced by high doses of insulin overcome the inhibitory effect of insulin on liver gluconeogenesis. In addition, the oral administration of sulphonylurea, like insulin injection, promote hypoglycemia and this effect was attributed to the insulin release. Thus, an expected effect of sulphonylurea induced hypoglycemia (SIH) would be activation of gluconeogenesis. However, sulphonylureas infused directly in the liver inhibits the gluconeogenesis, an this effect was mediated partially at least by an inactivation of mitochondrial function. **AIM** – Theregore, considering these opposite influences on liver gluconeogenesis we decided to clarify if the gluconeogenesis was activated or inhibited in livers from rats submitted to SIH. Since non diabetic rats is a suitable experimental model to investigate SIH and considering that *in situ* perfused livers reflects the *in vivo* conditions of the animal immediately before the liver isolation, this experimental approach was used. For this purpose glibenclamide, a sulphonylurea used in the treatment of type 2 diabetes was employed. **MATERIALS AND METHODS** - Male Wistar (180-220 g) 24 h fasted rats were used. The manipulation of the animals was approved by the ethical committee of the State University of Maringá, PR, Brazil. Thus, a preliminary experiment to characterize SIH after oral (intragastric) administration of glibenclamide (10 mg/kg) was done. Blood was obtained by decapitation. The values obtained for glycemia at 30, 60, 90, 120 and 150 min (means ± S.D, n = 6) after the oral administration of glibenlamide were 111.9 ± 13.68, 75.33 ± 4.5, 63.12 ± 3.04, 60.5 ± 2.74, and 71.96 ± 3.84 mg/dl, respectively. Thus, to verify the effect of oral SIH on liver gluconeogenesis, the experiments were done 2 h after glibenclamide administration (SIH group), when the lowest value of glycemia was obtained. Control normoglicemic group (Control group) was represented by animals which received saline. For liver perfusion experiments the rats were anaesthetized with thiopental (45 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* with Krebs Henseleit Bicarbonate (KHB). After a pre-infusion period (10 min with KHB), the gluconeogenic substrates (L-alanine, L-glutamine, L-lactate, pyruvate or glycerol) were dissolved in the perfusion fluid, followed by a post-infusion period (10 min only with KHB) to allow the return to basal

levels. Samples of the effluent perfusion fluid were collected at 5 min intervals and the liver production of glucose and urea were analyzed. The differences in the glucose and urea production during and before the infusion of the gluconeogenic substrate allowed to calculating the area under the curves (AUC). The addition of L-alanine, pyruvate, L-glutamine or glycerol increases the rate of glucose production proportionately to the amount of the glucose precursor until a saturating concentration is reached. Thus, by using a saturating concentration of liver glucose precursors it is possible to measure the maximal capacity of the liver to produce glucose from each gluconeogenic substrate. Thus, the gluconeogenic capacity in livers from rats which received oral glibenclamide (SIH group) or oral saline (Control group) were compared by using saturating concentration of L-alanine (5 mM), L-glutamine (5 mM), L-lactate (2 mM), pyruvate (5 mM) or glycerol (2 mM). **RESULTS -** In the first set of experiments livers from rats that received oral saline (Control group) or oral glibenclamide (SIH group) were infused with L-alanine (5 mM). Livers of SIH rats showed lower ($p < 0.05$) glucose and higher ($p < 0.05$) pyruvate production than livers of control rats. However, the urea and L-lactate production were similar. In the second set of experiments livers from control and SIH rats were infused with pyruvate (5 mM). Livers from SIH rats showed lower ($p < 0.05$) glucose and higher ($p < 0.05$) L-lactate production than livers from control rats. In the third set of experiments livers from control and SIH rats were infused with L-glutamine (5 mM). Livers from SIH rats showed lower ($p < 0.05$) glucose production than livers from control rats. However, the urea production was similar. In the fourth set of experiments livers from control and and SIH rats were infused with L-lactate (2 mM) or glycerol (2 mM). Livers from SIH rats showed lower ($p < 0.05$) glucose production from L-lactate than livers from control rats. However, the glucose production from glycerol (2 mM) was similar for both groups. In the fifth set of experiments livers from SIH rats were infused with physiological (0.45 mM) or saturating (5 mM) concentration of L-alanine. Livers infused with saturating concentration of L-alanine showed higher ($p < 0.05$) glucose and urea production than livers infused with physiological concentration of L-alanine. In the sixth set of experiments livers from SIH rats were infused with physiological (2 mM) and saturating (5 mM) concentrations of L-glutamine. Livers infused with saturating concentration of L-glutamine showed higher ($p < 0.05$) glucose and urea production than livers infused with physiological concentration of L-glutamine. **DISCUSSION** - The first gluconeogenic substrate investigated was L-alanine which cross the liver cell membrane and was then converted to pyruvate. From the cytosol, pyruvate enters the mitochondria. There, pyruvate was carboxylate and leaves

mitochondria as aspartate or malate. In the cytosol malate or aspartate was converted to oxalacetate, then to phosphoenolpyruvate and after various steps they were converted to glucose and released from the hepatocyte. Since this complex pathway depends of oxygen supply and several cellular compartments, the glucose, L-lactate and pyruvate production from L-alanine can be used as a marker of the integrity of hepatocyte. Therefore, by using saturating concentration of L-alanine we showed that the liver capacity to produce glucose during SIH was decreased ($p<0.05$). This result could not be attributed to the decreased catabolism of L-alanine, since the urea production from this amino acid was unchanged. Moreover, considering that the pyruvate production from L-alanine in livers from SIH rats was increased ($p<0.05$), the possibility of a lower entrance of pyruvate in the gluconeogenic pathway must be considered. In agreement with this proposition, liver from SIH rats showed a decreased ($p<0.05$) production of glucose not only from pyruvate but also from L-Lactate. Moreover, the liver glucose production from L-glutamine, that entered in the gluconeogenesis after the PC step, was also decreased ($p <0,05$). This result, could not be attributed to the decreased catabolism of L-glutamine, since the urea production from this amino acid was unchanged. However, the glucose production from glycerol which enter in the gluconeogenesis after the mitochondrial step was maintained. Taken together the results suggest that the inhibition of gluconeogenesis promoted by glibenclamide overcome the intensification of liver gluconeogenesis in response to hypoglycemia. Moreover, if these capabilities of glibenclamide occur *in vivo*, the result could be an intensification of the effectiveness of this drug as hypoglycemic agent. However, in spite of the decreased capacity to produce glucose during SIH, the possibility of the administration of gluconeogenic substrates during this condition must be considered. This affirmation was based in the fact that during SIH the liver glucose production from L-alanine and L-glutamine were lower. But maintained and influenced by the availability of these amino acids. Finally, our results and the considerations herein discussed, open the possibility of the administration of gluconeogenic substrates in the treatment of SIH, particularly when the therapy with glucose is not effective.

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Investigation of the gluconeogenic capacity in livers from rats submitted to glibenclamide induced hypoglycemia.

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Running title: Liver gluconeogenesis in rats submitted to glibenclamide induced hypoglycemia.

Investigation of the gluconeogenic capacity in livers from rats submitted to glibenclamide induced hypoglycemia.

Our previous studies demonstrate an increased liver capacity to produce glucose from several gluconeogenic substrates during insulin induced hypoglycemia (IIH). This effect was partly at least mediated by an increased release of counterregulatory hormones that overcome the inhibitory effect of insulin on liver gluconeogenesis (LG). Thus, an expected effect of sulphonylureas induced hypoglycemia (SIH), like IIH, was an activation of LG. But, sulphonylureas infused directly in the liver inhibits LG by an inhibition of the mitochondrial function. Thus, considering these opposite effects we investigated LG in non diabetic rats submitted to SIH. For this purpose 24 h fasted rats which received oral glibenclamide (10 mg/kg) were used (SIH group). Control group received oral saline. Glycemia at 30, 60, 90, 120 and 150 min (means ± S.D, n = 6) after the oral administration of glibenlamide were 111.9 ± 13.68 mg/dl, 75.33 ± 4.5 mg/dl, 63.12 ± 3.04 mg/dl, 60.5 ± 2.74 mg/dl, 71.96 ± 3.84 mg/dl, respectively. Considering that the lower glycemia was obtained 120 min after oral glibenclamide administration, this time was used to investigate LG in *situ* perfused livers. The results shown that the gluconeogenic capacity from substrates which enters in the gluconeogenesis before mitochondrial step, i.e., L-alanine (5 mM), L-lactate (2 mM), pyruvate (5 mM) and L-glutamine were decreased ($p < 0.05$). However, the gluconeogenic capacity from glycerol (2 mM), which enters in the gluconeogenesis after the mitochondrial step was maintained. Taken together the results suggest that the inhibition of liver gluconeogenesis promoted by oral glibenclamide could be attributed, partly at least to its effect on mitochondrial function.

Key words Glibenclamide; hepatic gluconeogenesis; hypoglycemia; rat

INTRODUCTION

In spite of the fact that physiological levels of insulin inhibits liver gluconeogenesis, supraphysiological levels of insulin which occur during insulin therapy[1)] stimulates gluconeogenesis. [2,3)] This paradoxical effect was explained by the fact that the counterregulatory hormones released during hypoglycemia induced by high doses of insulin overcome the inhibitory effect of insulin on liver gluconeogenesis.[4,5)]

In addition, the oral administration of sulphonylurea, like insulin injection, promote hypoglycemia and this effect was attributed to the insulin release. [6)] Thus, an expected effect of sulphonylurea induced hypoglycemia is an activation of gluconeogenesis.

However, it was well established that sulphonylureas infused directly in the liver inhibits the gluconeogenesis,[7)] an this effect was mediated partially at least by an inactivation of mitochondrial function.[8-10)]

Thus, considering these two opposite influences on liver gluconeogenesis we decided to clarify if the gluconeogenesis was activated or inhibited in livers from rats submitted to hypoglycemia induced by oral administration of sulphonylurea.

Since non diabetic rats is a suitable experimental model to investigate sulphonylurea induced hypoglycemia[7)] and considering that *in situ* perfused livers reflects the *in vivo* conditions of the animal immediately before the liver isolation[2,3)], this experimental approach was used. For this purpose glibenclamide, a sulphonylurea used in the treatment of type 2 diabetes was employed.

MATERIALS AND METHODS

Materials: Glibenclamide (also known as glyburide) was purchased from Sanofi Aventis. L-alanine, L-glutamine and pyruvate were obtained from Sigma-Aldrich Chemie. All other reagents were of the highest purity obtainable.

Animals: Adult male Wistar rats, weighting 180 - 220 g, were maintained on food and water *ad libitum* before all 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 8:00 a.m. All experiments were performed with 24 fasted rats (8:00 a.m – 8:00 a.m).

Experimental sulphonylurea induced hypoglycemia (SIH): A preliminary experiment to characterize the hypoglycemia after oral (intragastric) administration of glibenclamide (10 mg/kg) was done. Blood was obtained by decapitation. The values obtained for glycemia[11] at 30, 60, 90, 120 and 150 min (means ± S.D, n = 6) after the oral administration of glibenlamide were 111.9 ± 13.68 mg/dl, 75.33 ± 4.5 mg/dl, 63.12 ± 3.04 mg/dl, 60.5 ± 2.74 mg/dl, and 71.96 ± 3.84 mg/dl, respectively. Thus, to verify the effect of oral SIH on liver gluconeogenesis, the experiments were done 2 h after glibenclamide administration (hypoglycemic group), when the lowest value of glycemia was obtained. Control rats (normoglycemic group) were represented by animals which received oral saline instead glibenclamide.

Liver perfusion experiments: The rats were anaesthetized with an ip injection of sodium thiopental (45 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* according to the protocol previously described,[12] in which after a pre-perfusion period (10 min), the gluconeogenic substrate (L-alanine, L-glutamine, L-lactate, pyruvate or glycerol) 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 liver production of glucose[11] was analyzed. The differences in the glucose production during and before the infusion of the gluconeogenic substrate allowed

to calculate the area under the curves (AUC). In part of the experiments the liver production of urea[13] , pyruvate[14] and L-lactate[15] were evaluated.

Perfused livers from fasted rats produce negligible amounts of glucose in the absence of gluconeogenic precursors. The addition of L-alanine, pyruvate, L-glutamine or glycerol increases the rate of glucose production proportionately to the amount of the glucose precursor until a saturating concentration is reached. The saturating concentration for each substrate represents the lowest concentration at which the maximal glucose production was obtained (results not shown). Thus, by using a saturating concentration of liver glucose precursors it is possible to measure the maximal capacity of the liver to produce glucose from each gluconeogenic substrate. Thus, the gluconeogenic capacity in livers from rats which received oral glibenclamide (SIH group) or oral saline (Control group) were compared by using saturating concentration of L-alanine (5 mM), L-glutamine (5 mM), L-lactate (2 mM), pyruvate (5 mM) or glycerol (2 mM).

In part of the experiments, the role of the availability of the most important gluconeogenic amino acid, i.e., L-alanine[16] and the most abundant blood amino acid, i.e., L-glutamine[17] in the activation of gluconeogenesis in livers from hypoglycemic rats were investigated. For this purpose, saturating and physiological levels of these amino acids were compared. The physiological values of L-alanine and L-glutamine was obtained in a previous study[16].

Statistical analysis: Statistical analysis were done with the software Graph Pad Prism 5. Data concerning glycemia were analyzed by analysis of variance (ANOVA) followed by Tukey´s post-test. The results of liver perfusion experiments were analyzed by the unpaired Student's *t*-test. Values are reported as mean $\pm$ S.D. $p < 0.05$ was accepted for all comparisons.

RESULTS

In the first set of experiments livers from rats that received oral saline (normoglycemic rats) or oral glibenclamide (hypoglycemic rats) were infused with L-alanine (5 mM). Livers of hypoglycemic rats showed lower ($p < 0.05$) glucose (Fig. 1A) and higher ($p < 0.05$) pyruvate (Fig. 1C) production than livers of normoglycemic rats. However the urea (Fig. 1B) and L-lactate (Fig. 1D) production were similar.

In the second set of experiments livers from normoglycemic rats and hypoglycemic rats were infused with pyruvate (5 mM). Livers from hypoglycemic rats showed lower ($p < 0.05$) glucose (Fig. 2A) and higher ($p < 0.05$) L-lactate (Fig. 2B) production than livers from normoglycemic rats.

In the third set of experiments livers from normoglycemic rats and hypoglycemic rats were infused with L-glutamine (5 mM). Livers from hypoglycemic rats showed lower ($p < 0.05$) glucose production (Fig. 4A) than livers from normoglycemic rats. However, the urea production (Fig. 3B) was similar.

In the fourth set of experiments livers from normoglycemic rats and hypoglycemic rats were infused with L-lactate 2 mM (Fig. 3A) or glycerol 2 mM (Fig. 4B). Livers from hypoglycemic rats showed lower ($p < 0.05$) glucose production from L-lactate (Fig. 4A) than livers from normoglycemic rats. However, the glucose production from glycerol (2 mM) (Fig. 4B) was similar for both groups.

In the fifth set of experiments livers from hypoglycemic rats were infused with physiological (0.45 mM) or saturating (5 mM) concentration of L-alanine. Livers infused with saturating concentration of L-alanine showed higher ($p < 0.05$) glucose (Fig. 5A) and urea (Fig. 5B) production than livers infused with physiological concentration of L-alanine.

In the sixth set of experiments livers from hypoglycemic rats were infused with physiological (2.0 mM) and saturating (5 mM) concentrations of L-glutamine. Livers infused with saturating concentration of L-glutamine showed higher ($p < 0.05$) glucose (Fig. 6A) and urea (Fig. 6B) production than livers infused with physiological concentration of L-glutamine.

DISCUSSION

The first gluconeogenic substrate investigated was L-alanine which crossed the liver cell membrane and was then converted to pyruvate. From the cytosol, pyruvate enters the mitochondria. There, pyruvate was carboxylated and leaves mitochondria as aspartate or malate. In the cytosol malate was converted to oxalacetate, then to phosphoenolpyruvate and after of various steps they were converted by the microssomal glucose-6-phosphatase to glucose wich was and released from the hepatocyte (Fig. 7). Since this complex pathway depends of oxygen supply[8)] and several cellular compartments (plasma membrane, cytosol, mitochondria and microssomal fraction), the glucose, L-lactate and pyruvate production from L-alanine can be used as a marker of the integrity of the hepatocyte, since an absence of glucose production and/or high L-lactate:pyruvate ratio indicate low viability and/or poor oxygenation. Thus, L-alanine is a good indicator of the quality of the liver preparation.

Therefore, by using saturating concentration of L-alanine we showed that the capacity of the liver to produce glucose during SIH (Fig. 1A) was decreased ($p<0.05$). This result could not be attributed to the decreased catabolism of L-alanine, since the urea production from this amino acid was unchanged (Fig. 1B). Moreover, considering that the pyruvate production (Fig. 1C) from L-alanine in livers from SIH rats was increased ($p<0.05$), the

possibility of a lower entrance of pyruvate in the gluconeogenic pathway must be considered.

In agreement with this proposition, liver from SIH rats showed a decreased ($p<0.05$) production of glucose not only from pyruvate (Fig. 2A) but also from L-Lactate (Fig. 4A).

Moreover, the liver glucose production from L-glutamine, that entered in the gluconeogenesis after the Piruvate Carboxylase (PC) step, was also decreased (Fig. 3A). This result, could not be attributed to the decreased catabolism of L-glutamine, since the urea production from this amino acid was unchanged (Fig. 3B). However, the glucose production from glycerol which enter in the gluconeogenesis after the mitochondrial step was maintained.

Taken together the results suggest that the inhibition of gluconeogenesis promoted by glibenclamide overcome the intensification of liver gluconeogenesis in response to hypoglycemia[18-20] Moreover, if these capabilities of glibenclamide are exerced *in vivo*, the result will be an intensification of the effectiveness of this drug as hypoglycemic agent in the fasted state. Moreover, it is possible that this suggestion could be expanded to other sulphonylureas, specifically to fasted state. But not to fed state, since in this condition glibenclamide stimulates glycogenolysis [8] .

However, in spite of the decreased capacity to produce glucose during SIH, the possibility of the administration of gluconeogenic substrates during this condition must be considered. This affirmation was based in the fact that during SIH the hepatic glucose production from L-alanine (Fig. 5A) and L-glutamine (Fig. 6A) were lower but remained virtually maintained and influenced by the availability of these gluconeogenic substrates.

Finally, our results and the considerations herein discussed, open the possibility of the administration of gluconeogenic substrates in the treatment of SIH, particularly when the therapy with glucose is not effective.

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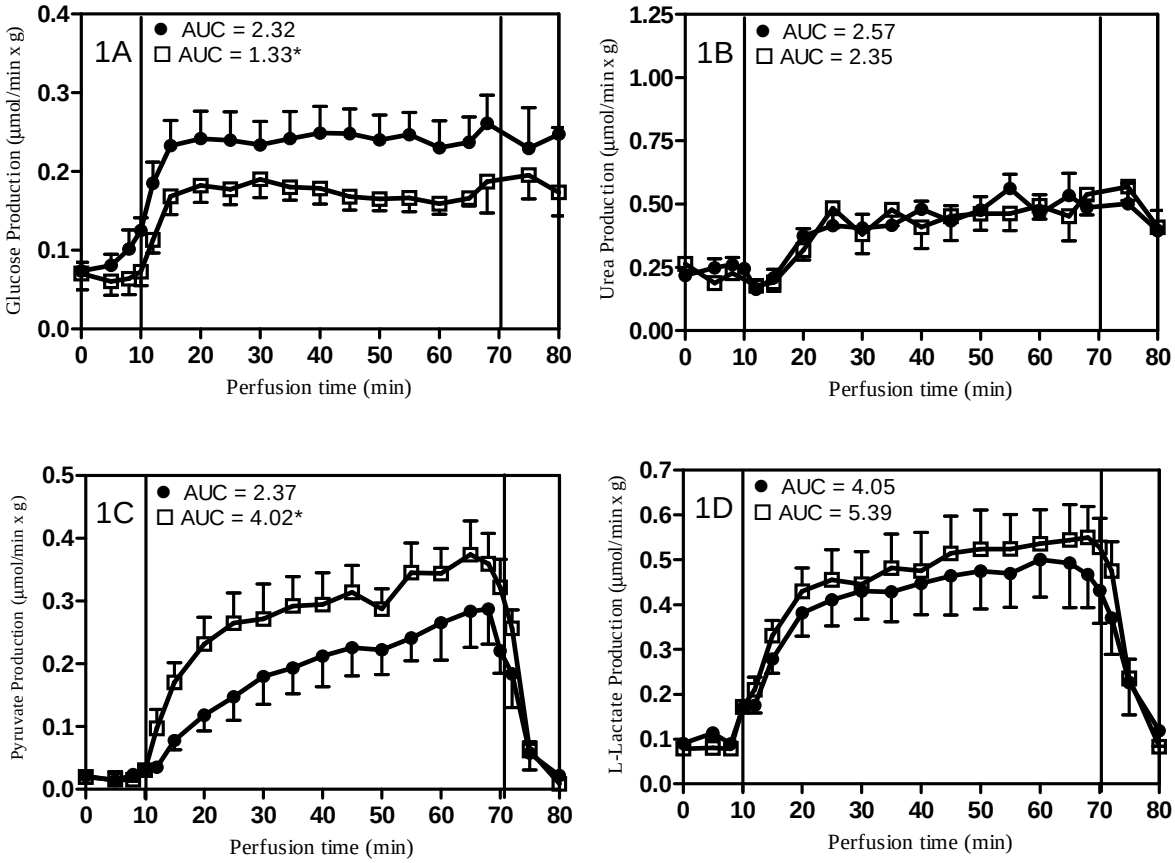

Fig.1. Glucose (A), urea (B), pyruvate (C) and L-lactate (D) production from L-alanine (5 mM) in perfused livers of 24 h fasted rats that received oral (10 mg/kg) glibenclamide (hypoglycemic, □) or saline (normoglycemic, ●) 2 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals and analyzed for glucose, urea, pyruvate and L-lactate. 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.

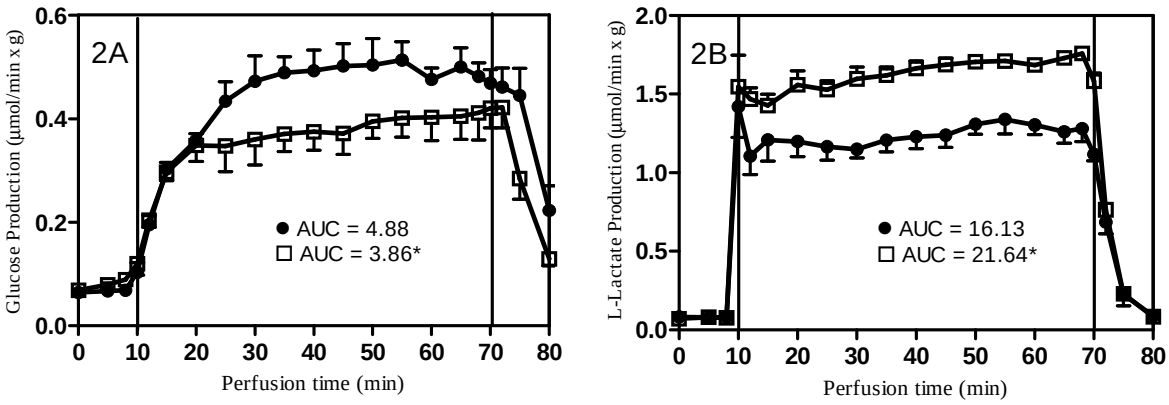

Fig. 2. Glucose (A) and L-lactate (B) production from pyruvate (5 mM) in perfused livers of 24 h fasted rats that received oral (10 mg/kg) glibenclamide (hypoglycemic,□) or saline (normoglycemic, ●) 2 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals and analyzed for glucose and L-lactate. 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.

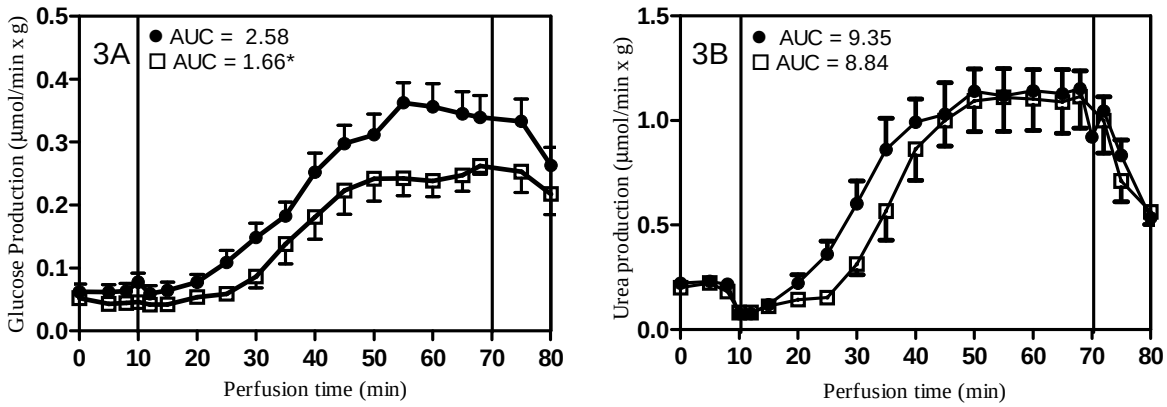

Fig. 3 Glucose (A) and urea (B) production from L-glutamine (5 mM) in perfused livers of 24 h fasted rats that received oral (10 mg/kg) glibenclamide (hypoglycemic, □) or saline (normoglycemic, ●) 2 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals and analyzed for glucose and urea. 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.

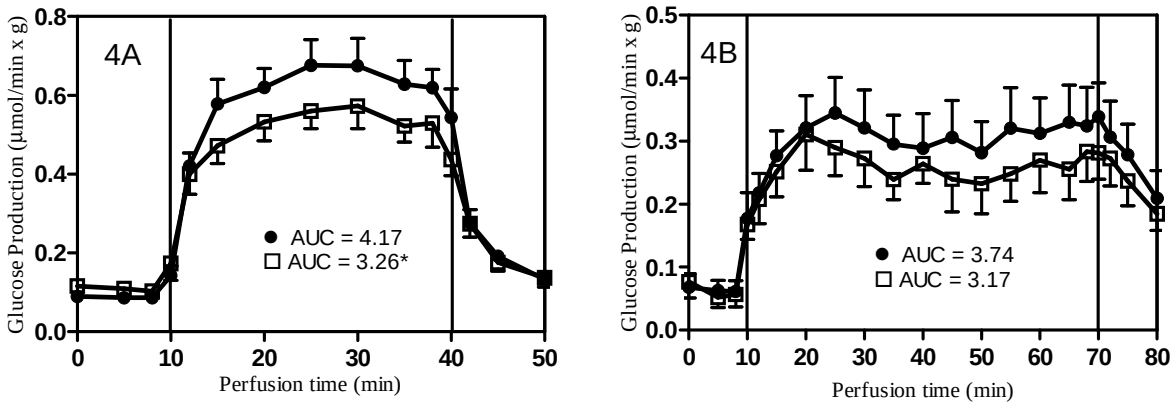

Fig. 4. Glucose production from L-lactate 2 mM (A) and glycerol 2 mM (B) in perfused livers of 24 h fasted rats that received oral (10 mg/kg) glibenclamide (hypoglycemic, □) or saline (normoglycemic, ●) 2 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals 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.

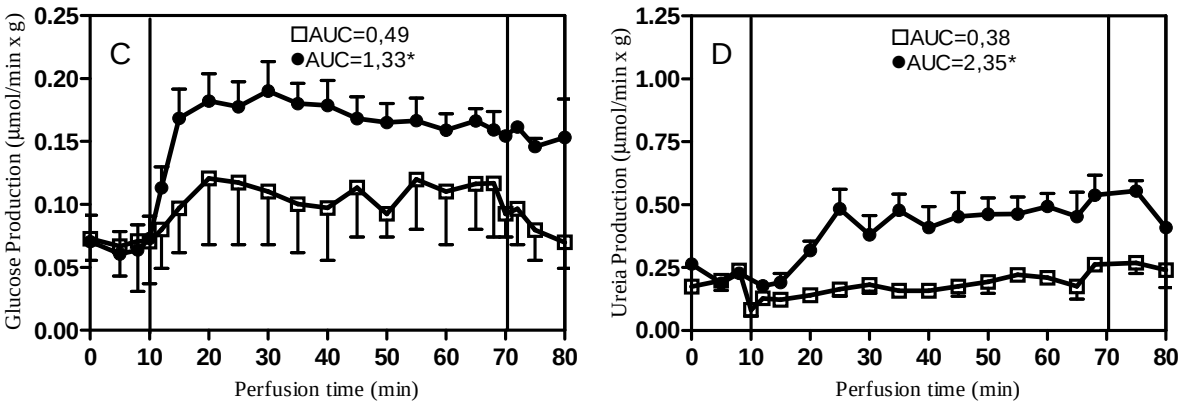

Fig. 5. Glucose (A) and urea (B) production from L-alanine (5 mM, ●) and L-alanine (0.45 mM, □) in perfused livers of 24 h fasted rats that received oral (10 mg/kg) glibenclamide (hypoglycemic) 2 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals and analyzed for glucose and urea. 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*. physiological value, i.e., 0.45 mM.

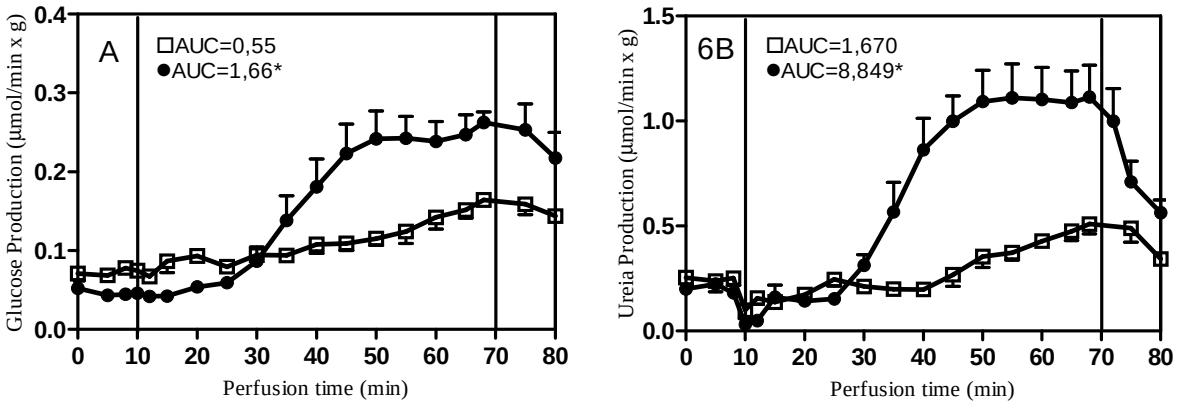

Fig. 6. Glucose (A) and urea (B) production from L-glutamine (5 mM, ●) and L-glutamine (2.0 mM, □) in perfused livers of 24 h fasted rats that received oral (10 mg/kg) glibenclamide (hypoglycemic) 2 h before the liver perfusion experiments. The effluent perfusate was sampled in 5 min intervals and analyzed for glucose and urea. 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*. physiological value, i.e., 2.0 mM.

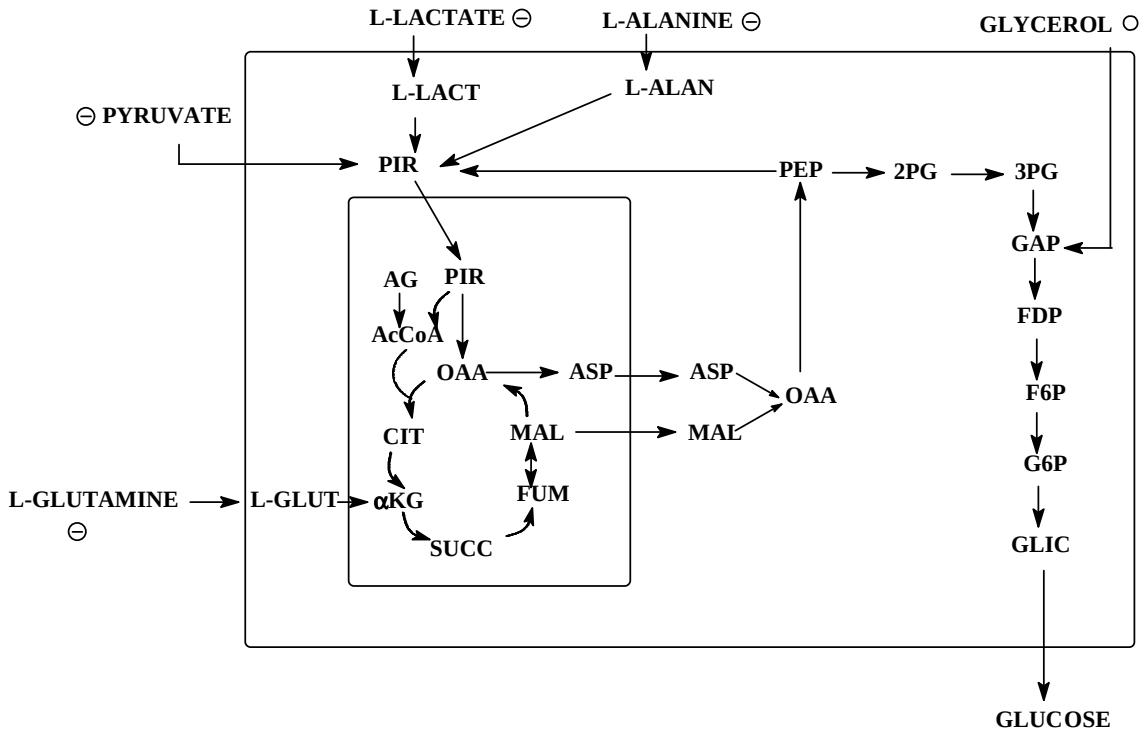

Fig. 7. Gluconeogenesis in the hepatocyte. Plasma membrane is represented by the greatest rectangle and mitochondria by the smallest rectangle. ⊖ Decreased gluconeogenesis, ○Maintained gluconeogenesis. Abbreviations: AcCoA, acetyl-CoA; ASP, aspartate; CIT, citrate; AG, fatty acid; FDP, fructose diphosphate; F6P, fructose 6-phosphate; FUM, fumarate; GAP, glyceraldehyde phosphate; G6P, glucose 6-phosphate; α-KG, α-ketoglutarate; L-Glut, L-glutamine; PYR, pyruvate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate, 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; SUCC, succinate.

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