GABRIELA GIRÃO DE ALBUQUERQUE

AVALIAÇÃO DA GLICONEOGÊNESE E CETOGÊNESE HEPÁTICA EM RESPOSTA À HIPOGLICEMIA DE CURTO PRAZO INDUZIDA POR INSULINA EM RATOS WISTAR EM JEJUM

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, área de concentração Biologia Celular, para obtenção do Título de Mestre.

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APRESENTAÇÃO DO TRABALHO

De acordo com as normas estabelecidas pela Coordenação do Programa de Pós-Graduação em Ciências Biológicas, esta tese de Mestrado foi redigida na forma de um artigo científico.

Gabriela Girão de Albuquerque, Vilma Aparecida Ferreira Godoi Gazola, Roberto Barbosa Bazotte. **Gluconeogenesis and ketogenesis in perfused liver of rats submitted to short term insulin induced hypoglycemia.** *Cell Biochemistry and Function.*

Dedico este trabalho:

aos meu pais, por terem acreditado em mim e por todo esforço que fizeram para possibilitar meus estudos,

aos meus irmãos pela colaboração para que isso fosse possível

ao Kléber por todo apoio, ajuda, companheirismo e por ter me ajudado a direcionar meu caminho

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RESUMO

Introdução

Para compreender os mecanismos de hipoglicemia induzida por insulina (HII) desenvolveu-se um modelo experimental no qual HII foi obtida com uma injeção intraperitoneal (ip) de insulina regular. Assim, utilizando este modelo experimental observou-se em um trabalho anterior uma aumentada gliconeogênese hepática e uma melhor recuperação da glicemia empregando L-alanina (Ala) ou L-glutamina (Gln). Além disso, como em trabalho anterior empregou-se apenas aminoácidos, neste estudo investigou-se três não aminoácidos precursores de glicose: glicerol (Gli), L-lactato (Lac) e piruvato (Pir).

Por outro lado, diante da inexistência de estudos abordando a capacidade hepática de produção de corpos cetônicos (CC) durante a HII, investigou-se no presente estudo a capacidade hepática de produção de CC, ou seja, a produção hepática de acetoacetato (ACE) + ß-hidroxibutirato (ßHB) durante a HII.

Material e Métodos

Ratos machos Wistar em jejum de 24-h foram empregados. HII foi obtida com uma injeção ip (1 U/kg) de insulina regular (Novolin®). O grupo controle normoglicêmico (Grupo COG) recebeu o mesmo volume de salina.

A concentração sanguínea de Gli, Lac, Pir e CC (ACE + ßHB) e os experimentos de perfusão foram realizados 30 min após a injeção de insulina (Grupo HII) ou salina (Grupo COG).

Considerando que a HII encontrava-se bem estabelecida 15 min após a injeção de insulina, este tempo foi selecionado para a administração oral de Gli (grupo HII + Gli), Lac (grupo HII + Lac), Pir (grupo HII + Pir) ou Gli + Lac + Pir (grupo HII + Gli + Lac + Pir). Além disso, dois grupos adicionais que receberam salina (grupo HII + SAL) ou glicose (grupo HII +

glicose) foram incluídos. Com exceção de salina, as doses de todas as demais substâncias, de 100 mg/kg, foram determinadas em um estudo anterior. A glicemia foi avaliada 15 min após a administração destas substâncias, ou seja, 30 min após a injeção de insulina. O sangue foi coletado por decapitação.

Os experimentos de perfusão de fígado foram antecedidos por anestesia obtida com injeção ip de tiopental (40 mg/kg) após a qual os ratos foram submetidos à laparotomia. A perfusão de fígado in situ foi realizada empregando se Krebs Henseleit, pH 7,4, saturado com O2/CO2. O fluido de perfusão foi impulsionado através de um oxigenador de membrana antes de entrar no fígado. Os experimentos foram executados de acordo com as seguintes fases. Após um período de pré-infusão (10 min), o substrato gliconeogênico foi dissolvido no fluido de perfusão e infundido entre 10 e 30 min do período de perfusão, seguido de um período de pós-infusão (10 min) para permitir o retorno à produção hepática basal de glicose (PHG). Amostras de fluido de perfusão foram coletadas a cada 5-min e determinou-se a concentração de glicose. As diferenças na produção de glicose durante (10-30 min) e antes (0-10 min) da infusão do substrato gliconeogênico permitiu calcular a área sobre a curva (ASC), expressa como µmol/g. Similar procedimento foi realizado quando concentração saturante de octanoato (0,3 mM) foi empregada como substrato cetogênico. CC foram obtidos pela soma dos valores de ACE + ßHB.

Obteve-se a capacidade e eficiência hepática em produzir glicose a partir de Gli, Lac ou Pir, através de experimentos nos quais empregou-se concentrações de Gli, Lac ou Pir a partir de valores sanguíneos basais a valores progressivamente mais elevados (2 mM, 5 mM, 10 mM, 20 mM until 40 mM) até se alcançar a concentração saturante de cada substratos. A adição de cada substratos gliconeogênicos elevou a taxa de PHG até que a concentração saturante de cada substrato foi alcançada, ou seja, a mais baixa concentração na qual a máxima PHG foi

alcançada. Esta abordagem experimental permite obter a capacidade e eficiência hepática em produzir glicose.

Resultados

A administração de insulina reduz a glicemia ($P< 0,05$) e eleva a concentração sanguínea de Lac e Pir ($P< 0,05$). Porém, a concentração sanguínea de Gli ou CC permaneceu inalterada.

A capacidade hepática em produzir glicose a partir de concentrações saturantes de Gli e Pir foram 5,0 mM (grupos COG e HII) e 10,0 mM (grupos COG e HII), respectivamente. Porém, para o Lac os valores obtidos foram 10,0 mM e 5,0 mM para os grupos COG e HII, respectivamente.

Ratos que receberam Lac ou Pir oral apresentaram elevação da glicemia ($P<0,05$), 15 min após a administração destas substâncias. Contudo, para o glicerol oral a elevação da glicemia ($P<0,05$) foi observada mais precocemente. Além disso, o melhor resultado foi obtido com a administração combinada de Gli + Lac + Pir; enquanto menor glicemia ($P<0,05$) foi obtida com a administração oral de glicose. Finalizando, a capacidade cetogênica, ou seja, a habilidade em produzir AC + ßHB a partir de concentração saturante de octanoato não foi influenciada pela injeção de insulina.

A máxima PHG a parte de Lac foi obtida com 50% da concentração usada para alcançar o mesmo efeito no fígado do grupo normoglicêmico. Assim, concluiu-se que eficiência hepática em produzir glicose a partir de concentração saturante de Lac estava elevada no grupo HII. Além disso, considerando o fato de que fígados provenientes de ratos HII apresentaram maior produção de glicose ($P < 0,05$) quando empregou-se concentração sanguínea basal de Pir, concluiu-se que a eficiência em produzir glicose a partir de concentração sanguínea basal de Pir estava intensificada em fígados provenientes de ratos HII. A maior eficiência em produzir glicose a partir de Lac e Pir em fígados provenientes de ratos HII ocorre provavelmente pelo

fato de que durante a HII a intensificada liberação de glucagon, adrenalina, hormônio do crescimento e cortisol sobrepujam o efeito inibitório da insulina na gliconeogênese.

Todavia, a eficiência hepática em produzir glicose a partir de Gli não foi modificada e uma possível explanação para este resultado é o fato de que o Gli entra na via gliconeogênica após as fases das quais participam as enzimas piruvato carboxilase e fosfoenolpiruvate carboxilase.

O fato de que a infusão de concentração supra fisiológica de Gli, Lac ou Pir produz mais glicose do que a obtida com a infusão de concentrações sanguíneas basais destas mesmas substancias, abre a possibilidade de que a administração oral de Gli, Lac ou Pir poderia auxiliar na recuperação da glicemia durante a HII. De acordo com esta sugestão, observou-se que a disponibilidade portal de substratos gliconeogênicos é relevante para a recuperação da glicemia. Esta conclusão baseia se no fato de que a melhor recuperação da glicemia foi obtida com a administração oral combinada de Gli, Lac e Pir. Em contraste, o pior resultado, obtido com administração oral de glicose, poderia ser imputado à possibilidade de uma interrupção na liberação de hormônios contra-reguladores acarretada por uma elevação transitória da glicemia após sua administração oral. De acordo com esta proposição, observou-se em estudo anterior uma inibição da gliconeogênese hepática em ratos HII que receberam glicose oral.

Além da gliconeogênese, investigou-se a capacidade hepática em produzir ACE + ß-HB a partir de concentração saturante de octanoato durante a HII. A concentração saturante de octanoato foi determinada previamente. Os resultados demonstraram que a despeito da hiperinsulinemia e do fato de a insulina inibir a cetogênese, a capacidade hepática de produção de CC foi mantida. De acordo com estes resultados obtidos em fígado isolado, a concentração sanguínea de CC não foi diferente (Grupo normoglicêmico vs. Grupo HII). Assim, é provável que durante a HII, a aumentada liberação de hormônios contra-reguladores também sobrepuja o efeito inibitório da insulina sobre a cetogênese hepática. Este efeito

poderia auxiliar na manutenção da glicemia em função de que uma limitada capacidade de gerar CC é favorável à HII.

Conclusão

Os resultados sugerem que em ratos submetidos à HII, a administração oral de Gli, Lac and Pir isoladamente ou em combinação promovem melhor recuperação da glicemia do que a administração oral de glicose. Contudo, considerando o efeito inibidor da PHG empregando alta concentração destas três substâncias, como previamente demonstrado com Ala e Gln, a aplicabilidade destes resultados necessita de estudos clínicos e experimentais mais aprofundados.

Palavras Chave: gliconeogênese, hipoglicemia, recuperação glicêmica, cetogênese, metabolismo hepático

ABSTRACT

Introduction

To understand the mechanisms of insulin induced hypoglycaemia (IIH) we developed an experimental model in which IIH was obtained with an intraperitoneal (ip) injection of regular insulin. Thus, by using this experimental model we observed in a previous work an increased hepatic gluconeogenesis and a better glycaemia recovery employing L-alanine (Ala) or L-glutamine (Gln). Since the previous work was done with amino acids we expanded the present study, investigating 3 non-amino acid glucose precursors: glycerol (Gly), L-lactate (Lac) and pyruvate (Pyr).

On the other hand, studies showing the effect of IIH on the liver capacity in producing ketone bodies (KB) are lacking. Therefore, in the present work we also investigated the hepatic capacity to produce KB, i.e., acetoacetate (ACE) + ßhydroxybutyrate (ßHB) during IIH.

Material and Methods

Male Wistar 24-h fasted rats were used. IIH was obtained with an ip injection (1 U/kg) of regular insulin (Novolin®). Control normoglycaemic rats (COG group) received an equal volume of saline.

The blood levels of Gly, Lac, Pyr and KB (ACE + ßHB) and liver perfusion experiments were done 30 min after insulin (IIH group) or saline (COG group) injection.

Since IIH was well-established 15 min after insulin injection, this time was selected to oral administration of Gly (IIH + Gly group), Lac (IIH + Lac group), Pyr (IIH + Pyr group) or Gly + Lac + Pyr (IIH + Gly + Lac + Pyr group). Moreover, two additional groups that received oral saline (IIH + SAL group) or glucose (IIH + glucose group) were included. Except saline, the dose of all these substances, i.e., 100 mg/kg, was determined in a previous study.

Glycemia were measured 15 min after the administration of these substances, i.e., 30 min after insulin injection. Blood was obtained from rats killed by decapitation.

For liver perfusion experiments, the rats were anaesthetised with an ip injection of thiopental (40 mg/kg) and submitted to laparotomy. The livers were perfused in situ using Krebs Henseleit, pH 7.4, saturated with O2/CO2. The perfusion fluid was pumped through a membrane oxygenator prior to entering the liver. The experiments were executed according the following steps. After a pre-perfusion period (10 min), the gluconeogenic substrate was dissolved in the perfusion fluid and infused between 10 and 30 min of the perfusion period, followed by a period of post-infusion (10 min) to allow the return to basal hepatic glucose production (HGP). Samples of the perfusion fluid were collected at 5-min intervals and the levels of glucose were determined. The differences in the glucose production during (10-30 min) and before (0-10 min) the infusion of the gluconeogenic substrate allowed calculate the area under the curves (AUC), expressed as μmol/g. Similar procedure was done when saturating level of octanoate (0.3 mM) was used as the ketogenic substrate. KB were obtained by the sum of ACE + ßHB values.

To obtain the liver capacity and efficiency to produce glucose from Gly, Lac or Pyr, experiments from basal blood levels of Gly, Lac or Pyr until saturating levels of each of these substrates (2 mM, 5 mM, 10 mM, 20 mM until 40 mM) were done. The addition of these gluconeogenic substrates increased the rate of HGP until the saturating level was reached, i.e., the lower concentration in which the maximal HGP was obtained. This experimental approach permits to obtain the liver capacity and efficiency to produce glucose.

Results

Insulin administration decreased ($P< 0.05$) glycaemia and increased the blood levels of Lac and Pyr ($P< 0.05$) but that of Gly and KB remained unchanged.

The liver capacity to produce glucose from a saturating levels of Gly and Pyr were 5.0 mM (COG and IIH group) and 10.0 mM (COG and IIH group), respectively, whereas for Lac the values obtained were 10.0 mM and 5.0 mM for COG and IIH group, respectively.

IIH rats that received oral Lac or Pyr showed increased ($P<0.05$) glycaemia, 15 min after the administration of these substances. However, to oral glycerol higher glycaemia ($P<0.05$) was observed earlier. In addition, the best result was obtained with combined administration of Gly + Lac + Pyr. In contrast, the lower glycaemia ($P<0.05$) was obtained with oral glucose. Finally, the ketogenic capacity, i.e., the ability to produce AC + ßHB from saturating level of octanoate was not influenced by insulin injection.

The maximal HGP from Lac was obtained with 50% of the concentration used to obtain the same effect in livers from COG group. It can be concluded that the liver efficiency to produce glucose from saturating levels of Lac was increased in the IIH group. Moreover, because livers of IIH rats showed higher ($P < 0.05$) glucose production with basal concentration of Pyr, we can conclude that the efficiency to produce glucose from basal concentration of Pyr was increased in livers of IIH rats. The higher efficiency to produce glucose from Lac and Pyr in livers of IIH rats occurs probably because during IIH the increased release of glucagon, epinephrine, grow hormone and cortisol overcome the inhibitory effect of insulin on gluconeogenesis.

However, the liver efficiency to produce glucose from Gly was not modified and a possible explanation for this result is the fact that Gly enters in the gluconeogenic pathway after the pyruvate carboxylase and phosphoenolpyruvate carboxylase step.

The fact that the infusion of supraphisiological concentrations of Gly, Lac and Pyr produce more glucose than that obtained with the infusion of basal concentration of the same substances, open the possibility that the oral administration of Gly, Lac and Pyr could help

glucose recovery during IIH. In agreement, with this suggestion, we observed that the portal availability of gluconeogenic substrates is important to glycemia recovery. This conclusion is based in the fact that the best glucose recovery was obtained with the combined oral administration of Gly, Lac and Pyr. In contrast, the worse result, obtained with oral administration of glucose, could be imputed to the possibility of a blunt in the release of counterregulatory hormones promoted by a transitory elevation of glycaemia after its oral administration. In agreement, we observed an inhibition of liver gluconeogenesis in IIH rats which received oral glucose. In addition to gluconeogenesis, we investigated the liver capacity to produce ACE + ß-HB from a saturating level of octanoate during IIH. The saturating level of octanoate was previously determined. The results showed that in spite of hyperinsulinemia and the fact that insulin inhibits ketogenesis, the capacity of the liver to produce KB was maintained. In agreement with the results obtained in isolated liver, the blood levels of KB were not different (COG group vs. IIH group). Thus, it seems that during IIH the increased release of counter-regulatory hormones also overcome the inhibitory effect of insulin on hepatic ketogenesis. This effect could help glycaemia maintenance because a limited capacity to generate KB is favourable to IIH.

Conclusion

The results suggest that oral Gly, Lac and Pyr alone or in combination are better than glucose for promoting glycaemic recovery in IIH rats. However, considering the inhibition of the HGP with high concentration of these substances, as previously demonstrate with Ala and Gln, the applicability of these results needs further experimental and clinical studies.

Key Words: gluconeogenesis, hypoglycaemia, glycaemia recovery, ketogenesis, liver metabolism.

Gluconeogenesis and ketogenesis in perfused liver of rats submitted to short term insulin induced hypoglycaemia

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Running Title: Gluconeogenesis and ketogenesis in rats submitted to hypoglycemia

Key Words: gluconeogenesis, ketogenesis, hypoglycaemia, glycerol, L-lactate, pyruvate

ABSTRACT

Gluconeogenesis and ketogenesis in perfused liver of rats submitted to short-term insulin induced hypoglycaemia (IIH) were investigated. For this purpose, 24-h fasted rats that received intraperitoneal (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. For gluconeogenesis studies, livers from IIH and COG groups were perfused with increasing concentrations (from basal blood concentrations until saturating concentration) of glycerol (Gly), L-Lactate (Lac) or pyruvate (Pyr). Livers of IIH group showed maintained efficiency to produce glucose from Gly and higher efficiency to produce glucose from Lac and Pyr. In agreement with these results the oral administration of Gly (100 mg/kg), Lac (100 mg/kg), Pyr (100 mg/kg) or Gly (100 mg/kg) + Lac (100 mg/kg) + Pyr (100 mg/kg) promoted glycaemia recovery. It can be inferred that the increased portal availability of Lac, Pyr and Gly could help glycaemic recovery by a mechanism mediated, partly at least, by a maintained (Gly) or increased (Lac and Pyr) hepatic efficiency to produce glucose. Moreover, in spite the fact that insulin inhibits ketogenesis, the capacity of the liver to produce ketone bodies from octanoate during IIH was maintained.

Key Words: gluconeogenesis, hypoglycaemia, glycaemia recovery, ketogenesis, liver metabolism

Introduction

It is well established that insulin at physiological levels inhibits key enzymes of gluconeogenesis, ketogenesis and the mobilization of gluconeogenic and ketogenic substrates to the liver during the fasting[1,2]. Therefore, low blood levels of insulin represent a crucial mechanism to maintain glycaemia during fasting. However, a condition of insulin induced hypoglycaemia (IIH) associated with fasting could be observed in diabetic patients submitted to insulin therapy[3]. Since IIH is the main limitation to the implementation of an intensive insulin schedule[4] which is necessary to prevent chronic complications[5], the investigation of the mechanism by which hypoglycaemia occurs is necessary.

To understand better the mechanisms of IIH we developed an experimental rat model in which hypoglycaemia was obtained by an intraperitoneal injection of a pharmacological dose of regular insulin[6-10]. Thus, by using this experimental model we observed in a previous work[11] an increased hepatic gluconeogenesis and a better glycaemia recovery employing the most important gluconeogenic amino acid, L-alanine[12] and the most abundant extracellular amino acid, L-glutamine[13]. Since the previous work[11] was done with amino acids we expanded this study, investigating three very important non-amino acid glucose precursors, i.e., glycerol, L-lactate and pyruvate.

On the other hand, studies showing the effect of IIH on the liver capacity in producing ketone bodies are lacking. Therefore, in the present work we also investigated the hepatic capacity to produce acetoacetate and ß-hydroxybutyrate during IIH.

Material and methods

Materials

Regular insulin (Novolin®) was purchased from Novo Nordisk (São Paulo, Brazil). β-hydroxybutyrate dehydrogenase was obtained from Sigma Chemical Company (St. Louis, USA). Glycerol (Gly), L-lactate (Lac), pyruvate (Pyr) and all other reagents were of the highest purity obtainable.

Animals

Male Wistar 24-h fasted rats weighing about 200 g were used in this study. The rats were maintained under constant temperature (23°C) with automatically controlled photoperiod (12-h light/12-h dark). All animals were food deprived from 8:00 a.m and the insulin induced hypoglycaemia (IIH) protocol started 24-h later. The manipulation followed the Brazilian law on the protection of animals.

Insulin induced hypoglycaemia (IIH)

IIH was obtained with an intraperitoneal (ip) injection of regular insulin (1 U/kg). Control normoglycaemic rats (COG group) received an equal volume of saline. Blood was obtained from rats killed by decapitation 30 min after insulin or saline administration. In addition to glycaemia[14], blood levels of Gly[15], Lac[16], Pyr[17] and ketone bodies were measured (Table 1). Ketone bodies were obtained by the sum of acetoacetate[18] plus ß-hydroxybutyrate[19] values.

Effect of oral administration of gluconeogenic precursors on glycaemia recovery during insulin induced hypoglycaemia (IIH)

Since IIH was well-established 15 min after insulin injection (not shown), this time was selected to oral administration of Gly (IIH + Gly group), Lac (IIH + Lac group), Pyr (IIH + Pyr group) or Gly + Lac + Pyr (IIH + Gly + Lac + Pyr group). Moreover, two additional

groups that received oral saline (IIH + saline group) or glucose (IIH + glucose group) were included. Except saline, the dose of all these substances, i.e., 100 mg/kg, was determined in a previous study[9]. Blood levels of glucose were measured 15 min after the administration of these substances, i.e., 30 min after insulin administration. Blood was obtained from rats killed by decapitation.

Liver perfusion technique

The animals were anaesthetised with an intraperitoneal injection of sodium thiopental (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), the gluconeogenic substrate was dissolved in the perfusion fluid and infused between the 10th and 30th 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 concentrations of glucose[14] were determined. The differences in the glucose production during (10-30 min) and before (0-10 min) the infusion of the gluconeogenic substrate allowed calculate the area under the curves (AUC), expressed as µmol/g. Similar procedure was done when saturating concentration of octanoate (0.3 mM) was used as the ketogenic substrate. The saturating concentration of octanoate was previously determined[20]. Ketone bodies were obtained by the sum of acetoacetate[18] plus ß-hydroxybutyrate[19] values.

Thus, the AUCs showed in Table 2 were obtained from similar experiments as that illustrated in Fig. 1.

Determination of the liver capacity and efficiency to produce glucose from glycerol, L-lactate and pyruvate

Liver perfusion experiments from basal blood concentrations of Gly, Lac or Pyr (as shown in Table 1) until saturating concentration of each of these substrates (2 mM, 5 mM, 10 mM, 20 mM until 40 mM) were employed. The addition of these gluconeogenic substrates increased the rate of glucose production until the saturating concentration was reached, i.e., the concentration in which the maximal hepatic glucose production was obtained (Table 2). This experimental approach permits to obtain the liver capacity and efficiency to produce glucose.

Statistical analysis

Statistical analysis were performed using unpaired Student's t-test or ANOVA followed by Tukey's post-test, using the Graph-Pad Prism program (version 3.0). The data were presented as means ± standard deviation of the means (SD). $P < 0.05$ was considered statistically significant.

Results

Insulin administration decreased ($P < 0.05$) the blood levels of glucose and increased the blood levels of Lac and Pyr ($P < 0.05$) but that of Gly and ketone bodies remained unchanged (Table 1).

The liver capacity (defined as the lower concentration in which the maximal glucose production was obtained) from increasing concentrations of Gly, Lac and Pyr 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 Gly and Pyr were 5.0 mM (COG and IIH group) and 10.0 mM (COG and IIH group), respectively, whereas for Lac the values obtained were 10.0 mM and 5.0 mM for COG and IIH group, respectively (Table 2).

The results obtained with IIH rats that received oral Lac or Pyr showed increased ($P<0.05$) glycaemia, 15 min after the administration of these substances. However, higher glycaemia ($P<0.05$) with oral glycerol was observed until 8 min, but not 15 min after the administration of this hepatic glucose precursor (results not shown). In addition, the best result was obtained with combined administration of Gly + Lac + Pyr. In contrast, the lower glycaemia ($P<0.05$) was obtained with oral glucose (Table 3).

Finally, the ketogenic capacity, i.e., the ability to produce acetoacetate plus ß-hydroxybutyrate from saturating concentration of octanoate (Table 4) was not influenced by insulin administration (IIH group *vs.* COG group).

Discussion

The present work used isolated livers from 24-h fasted rats, a favourable condition for gluconeogenesis[11, 20] and ketogenesis[21] in which hepatic glycogen was depleted. The major advantage of using isolated livers is that no extra hepatic effects such as increased sympathetic activity and/or endogenous hormone influence the results[22, 23].

Thus, by using isolated perfused liver we determined the hepatic capacity and efficiency to produce glucose employing increasing concentrations of Gly, Lac and Pyr. The addition of these glucose precursors increased the rate of glucose production until the liver capacity was reached, i.e., the lowest concentration at which the maximal hepatic glucose production was obtained (Table 2). The maximal glucose production from Lac was obtained

with 50% of the concentration used to obtain the same effect in livers from COG group. It can be concluded that the liver efficiency to produce glucose from saturating levels of Lac was increased in the IIH group (Table 2). Moreover, because livers of IIH rats showed higher ($P < 0.05$) glucose production with basal blood concentration of pyruvate, we can conclude that the efficiency to produce glucose from basal concentration of pyruvate was increased in livers of IIH rats.

The higher efficiency to produce glucose from Lac and Pyr in livers of IIH rats occurs probably because during IIH the increased release of counter-regulatory hormones, i.e., glucagon[24], epinephrine[25], grow hormone[26] and cortisol[27] overcome the inhibitory effect of insulin on hepatic gluconeogenesis[1]. However, the liver efficiency to produce glucose from Gly was not modified (Table 2). In the case of Gly, a possible explanation for this result is the fact that Gly enters in the gluconeogenic pathway after the pyruvate carboxylase and phosphoenolpyruvate carboxylase step[1,2].

The fact that the infusion of supraphysiological concentrations of Gly, Lac and Pyr (Table 2) produce more glucose than that obtained with the infusion of basal blood concentration (Table 1) of the same substances, open the possibility that the oral administration of Gly, Lac and Pyr could help glucose recovery during IIH.

In agreement with this suggestion, we observed that the portal availability of gluconeogenic substrates is important to glycemia recovery. This conclusion is based in the fact that the best glucose recovery was obtained with the combined oral administration of Gly, Lac and Pyr. On the other hand, the worse result, obtained with oral administration of glucose, could be imputed to the possibility of a blunt in the release of counterregulatory hormones promoted by a transitory elevation of glycaemia after its oral administration (results not

shown). In agreement, we observed an inhibition of hepatic gluconeogenesis in IIH rats which received oral glucose (data not shown).

In addition to gluconeogenesis, we investigated the hepatic capacity to produce acetoacetate and ß-hydroxybutyrate from a saturating concentration of octanoate during IIH. The saturating concentration of octanoate was previously determined[20]. The choice of a medium chain fatty acid as ketogenic precursor was based in the following facts[28] : 1) octanoate is water soluble; 2) its transport across the mitochondrial membrane does not require carnitine acyltransferase action; 3) octanoate is exclusively metabolized by the mitochondria; 4) octanoate shows higher capacity to produce ketone bodies if compared with estearate, palmitate, miristate, laureate and decanoate.

The results showed that in spite of hyperinsulinemia[7] and the fact that insulin inhibits ketogenesis[20], the capacity of the liver to produce ketone bodies was maintained (Table 4) in IIH rats. In agreement with the results obtained in isolated liver, the blood glucose levels of ketone bodies (Table 1) were not different (COG group *vs.* IIH group). Thus, it seems that during IIH the increased release of counter-regulatory hormones[24-27] also overcome the inhibitory effect of insulin on hepatic ketogenesis[1]. This effect could contribute to glycaemia maintenance because a limited capacity to generate ketone bodies seems to be favourable to hypoglycaemia. For example, the occurrence of hypoketotic hypoglycemia in children as consequence of several modalities of fatty acid oxidation disorders has demonstrated to be associated with a limited hepatic capacity to generate ketone bodies[29].

Finally, the results suggest that oral Gly, Lac and Pyr alone or in combination are better than glucose for promoting glycaemic recovery in IIH rats. However, considering the inhibition of the liver glucose production with high concentration of these substances (Table

2), as was also previously demonstrate with L-alanine and L-glutamine[11], the applicability of these results needs further experimental and clinical studies.

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Table 1

Basal blood levels of glucose, glycerol, L-lactate, pyruvate and ketone bodies, 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 of six to eight experiments.

Basal blood levels (mM)	**COG group**	**IIH group**
Glucose	**4.84 ± 0.28**	**2.29 ± 0.18***
Glycerol	**0.144 ± 0.004**	**0.145 ± 0.014**
L-Lactate	**2.211 ± 0.162**	**2.653 ± 0.199***
Pyruvate	**0.450 ± 0.082**	**0.546 ± 0.089***
Ketone Bodies	**0.053 ± 0.002**	**0.055 ± 0.004**

***P < 0.05 vs. COG**

Table 2

Glucose production from increasing concentrations of glycerol, L-lactate and pyruvate in perfused livers of normoglycaemic (COG group) and hypoglycaemic (IIH group) 24 h-fasted rats. Basal blood levels of glycerol, L-lactate and pyruvate were previously described (Table 1). The areas under the curves (AUC) were obtained as described in Materials and Methods and Figure 1. The AUC values (μmol/g) were expressed as means + SD of six to-eight liver perfusion experiments. * P < 0.05 if compared with the highest value in the same column. Nd, not determined.

	Glucose production (μmol/g)					
	Glycerol		**L-lactate**		**Pyruvate**	
	COG	**IIH**	**COG**	**IIH**	**COG**	**IIH**
Blood levels	**3.9 ± 0.2***	**4.1 ± 0.2***	**5.3 ± 0.1***	**4.1 ± 0.1***	**3.7 ± 0.2***	**6.7 ± 0.1***
2 mM	**4.6 ± 0.2***	**4.6 ± 0.2***	**Nd**	**Nd**	**4.9 ± 0.1***	**6.8 ± 0.2q***
5 mM	**5.6 ± 0.7**	**5.7 ± 0.3**	**4.7 ± 0.5***	**6.9 ± 0.5**	**6.6 ± 0.4***	**6.5 ± 0.3***
10 mM	**4.3 ± 0.0***	**4.3 ± 0.6***	**7.3 ± 0.2**	**3.4 ± 0.1***	**10.5 ± 0.4**	**8.2 ± 0.5**
20 mM	**Nd**	**Nd**	**3.4 ± 0.4***	**3.1 ± 0.3***	**7.6 ± 0.1***	**8.6 ± 0.5**
40 mM	**Nd**	**Nd**	**Nd**	**Nd**	**5.7 ± 1.3***	**7.1 ± 0.6***

Table 3

Effect of saline (IIH + saline group), glucose (IIH + glucose group), glycerol (IIH + Gly group), L-lactate (IIH + Lac group), pyruvate (IIH + Pyr group) or Gly+Lac+Pyr (IIH + Gly+Lac+Pyr group) on glycaemia during insulin-induced hypoglycaemia (IIH). All substances were orally administered 15 min after insulin injection (1 U/kg). Glycaemia was determined 15 min later, i.e. 30 min after insulin injection. The results were expressed as means ± SD of six to eight experiments.

Groups	**Glycaemia (mM)**
IIH + Saline	**2.91 ± 0.1**
IIH + Glucose	**2.65 ± 0.1***
IIH +Glycerol	**2.90 ± 0.1**
IIH +Pyruvate	**3.12 ± 0.2***
IIH + L-lactate	**3.13 ± 0.1***
IIH + Gly+Lac+Pyr	**3.40 ± 0.1***

$^*P < 0.05$ vs. IIH + saline group

Table 4

Ketone bodies production from saturating concentration of octanoate in livers from normoglycaemic (COG group) and hypoglycaemic (IIH groups) 24 h-fasted rats. The areas under the curves (AUC), obtained as described in Materials and Methods, were expressed as means ± SD of six to eight-liver perfusion experiments.

Ketogenic substrate	Ketone bodies production (µmol/g)	
Octanoate (0.3 mM)	COG	IIH
	54.7 ± 15.5	53.3 ± 20.1

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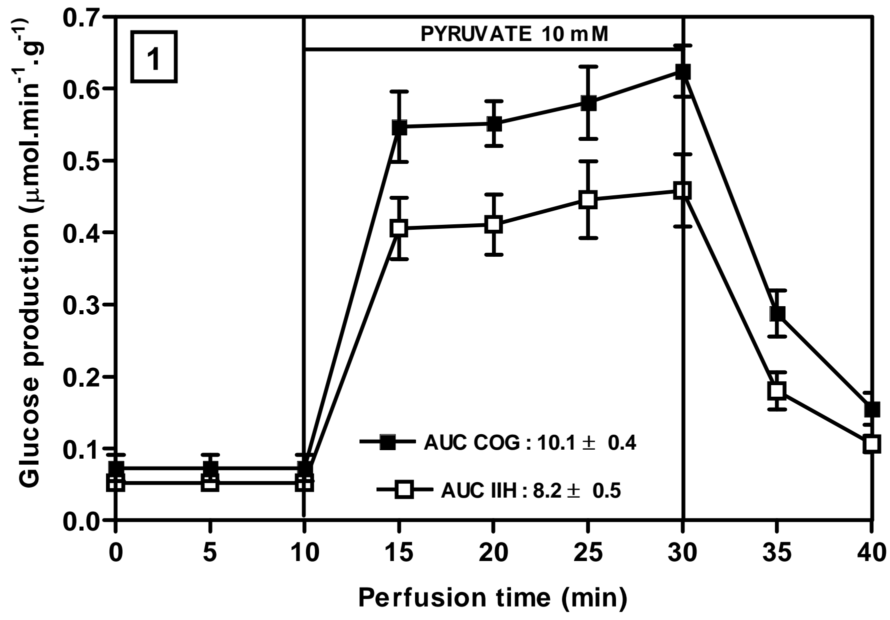
Fig. 1. Demonstrative experiments. Time courses of glucose production from saturating concentration of pyruvate in perfused livers from 24-h fasted rats. The effluent perfusate was sampled in 5 min intervals and determined for glucose. The area under the curves (AUC), expressed as μmol/g, were obtained from the difference between the values during and before pyruvate infusion. The results were expressed as means ± SD of six to eight-liver perfusion experiments.


1
PYRUVATE 10 mM
Glucose production (µmol.min-1.g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
AUC COG : 10.1 ± 0.4
AUC IIH : 8.2 ± 0.5
0
5
10
15
20
25
30
35
40
Perfusion time (min)

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