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Participation of the liver gluconeogenesis in the glibenclamide-induced hypoglycaemia in rats

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We previously demonstrated an increased liver gluconeogenesis (LG) during insulin-induced hypoglycaemia. Thus, an expected effect of sulphonylureas induced hypoglycaemia (SIH) could be the activation of LG. However, sulphonylureas infused directly in to the liver inhibits LG. Considering these opposite effects we investigated herein LG in rats submitted to SIH. For this purpose, 24 h fasted rats that received glibenclamide (10 mg kg^{-1}) were used (SIH group). Control group received oral saline. Glycaemia at 30, 60, 90, 120 and 150 min after oral administration of glibenclamide were evaluated. Since the lowest glycaemia was obtained 120 min after glibenclamide administration, this time was chosen to investigate LG *in situ* perfused livers. The gluconeogenesis from precursors that enters in this metabolic pathway before the mitochondrial step, i.e. L-alanine (5 mM), L-lactate (2 mM), pyruvate (5 mM) and L-glutamine were decreased ($p < 0{\cdot}05$). However, the gluconeogenic activity using glycerol (2 mM), which enters in the gluconeogenesis after the mitochondrial step was maintained. Taken together, the results suggest that the inhibition of LG promoted by SIH overcome the activation of this metabolic pathway promoted by IIH and could be attributed, at least in part, to its effect on mitochondrial function. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS — sulphonylureas; gluconeogenesis; liver metabolism; hypoglycaemia; glycaemia control; mitochondrial metabolism; glutamine; alanine

INTRODUCTION

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

In addition, the oral administration of sulphonylurea also promotes hypoglycaemia and the effect was consequence of the insulin release.[6] Thus, an expected effect of sulphonylurea induced hypoglycaemia is an activation of gluconeogenesis.

However, it is well established that sulphonylureas infused directly in the liver inhibits the gluconeogenesis,[7] an this effect is partially mediated 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, inhibited or maintained in livers from rats submitted to hypoglycaemia induced by oral administration of sulphonylurea.

Since non-diabetic rats are suitable experimental model to investigate sulphonylurea-induced hypoglycaemia[6] 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 (Sao Paulo, Brazil). L-alanine, L-glutamine and pyruvate were obtained from ICN Company (Costa Mesa, California, USA). The majority of other chemicals were obtained from Sigma-Aldrich Brazil Ltda (Sao Paulo, Brazil).

Animals

Adult male Wistar rats, weighting 180–220 g, were maintained on food and water *ad libitum* before all experimental procedures. The manipulation of animals was approved by the ethical committee of the State University of Maringá, PR, Brazil. On the day before the

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experiment, the animals were food deprived from 8:00 AM (8:00 AM–8:00 AM). All experiments were done with 24 h fasted rats.

Sulphonylurea induced hypoglycaemia (SIH)

A preliminary experiment to characterize SIH after oral (intragastric) administration of glibenclamide (10 mg kg^{-1}) was done. The dose of sulphonylurea was based on a previous study.[6] Blood was collected by decapitation at 30, 60, 90, 120 and 150 min after the administration of glibenlamide and the values (mean ± S.D, $n = 6$) of glycaemia[11] were $111{\cdot}9 \pm 13{\cdot}7$, $75{\cdot}3 \pm 4{\cdot}5$, $63{\cdot}1 \pm 3{\cdot}0$, $60{\cdot}5 \pm 2{\cdot}7$ and $72{\cdot}0 \pm 3{\cdot}8$ mg dl^{-1}, respectively. Since the lowest glycaemia was obtained 120 min after glibenclamide administration, this time was used to investigate liver gluconeogenesis *in situ* perfused livers of SIH rats (hypoglycaemic group). Control rats (normoglycaemic group) were represented by animals which received oral saline instead glibenclamide.

Liver perfusion experiments

The rats were anaesthetized 120 min after glibenclamide or saline administration with an IP injection of sodium thiopental (45 mg kg^{-1}) 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 glucose[11] production was measured. The differences in the glucose production during and before the infusion of the gluconeogenic substrate allowed calculating the area under the curves (AUC). In part of the experiments the urea,[13] pyruvate[14] and L-lactate[15] production were evaluated.

Perfused livers from fasted rats release 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 until a saturating concentration was reached, i.e. 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 release glucose from each gluconeogenic substrate. Thus, the gluconeogenic capacity in livers from rats that received oral glibenclamide (hypoglycaemic group) or oral saline (Control group) were compared by using saturating concentration

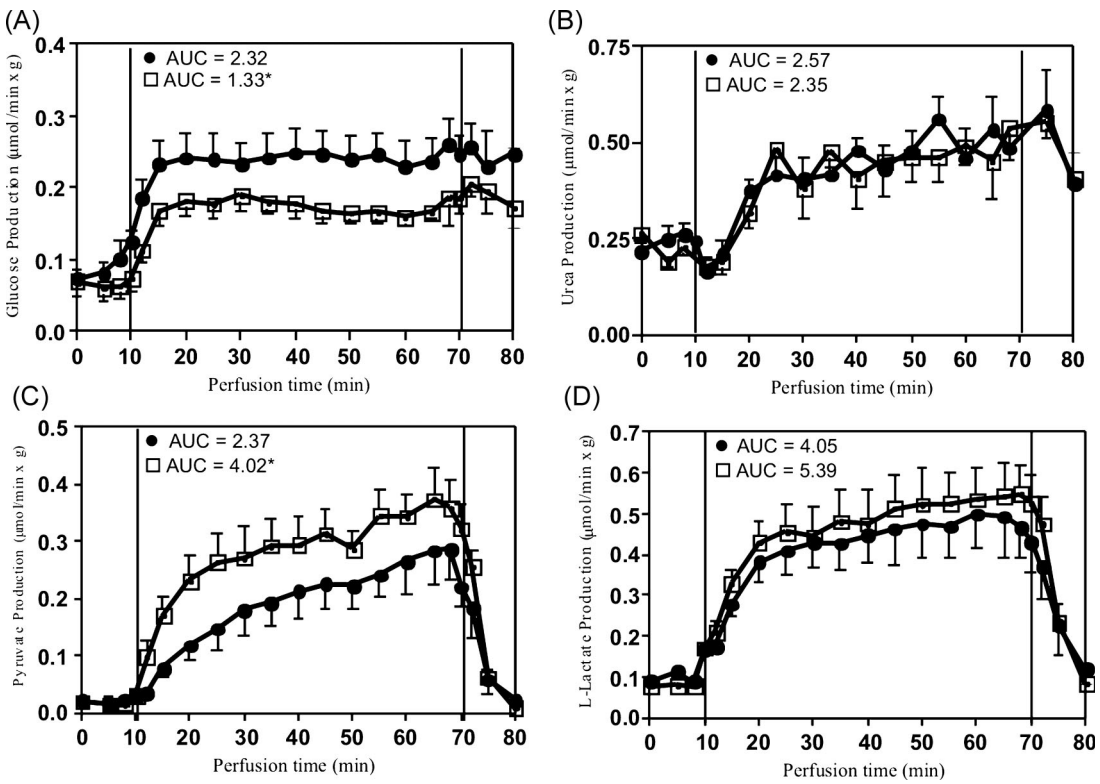

Figure 1. Glucose (A), urea (B), pyruvate (C) and L-lactate (D) production from L-alanine (5 mM) in perfused livers of 24 h fasted rats that received oral (10 mg kg^{-1}) glibenclamide (hypoglycaemic, □) or saline (normoglycaemic, ●) 120 min before the liver perfusion experiments. L-alanine was infused between 10 and 70 min. The effluent perfusate was sampled in 5 min intervals and analysed for glucose, urea, pyruvate and L-lactate. The AUC, areas under the curves (µmol g^{-1}) were obtained as described in Materials and Methods section. Data were expressed as mean ± SD of four individual liver perfusion experiments. $^{*}p < 0{\cdot}05$ versus normoglycaemic group

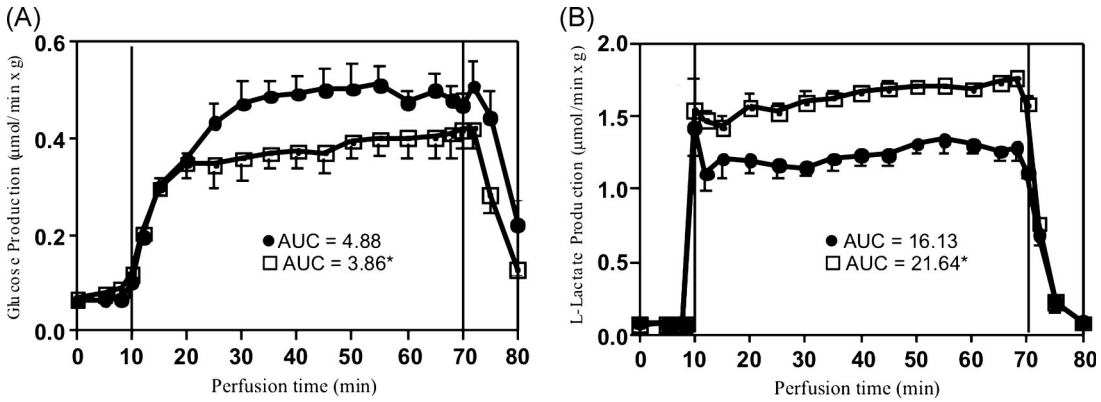

Figure 2. Glucose (A) and L-lactate (B) production from pyruvate (5 mM) in perfused livers of 24 h fasted rats that received oral (10 mg kg^{-1}) glibenclamide (hypoglycaemic, □) or saline (normoglycaemic, ●) 120 min before the liver perfusion experiments. Pyruvate was infused between 10 and 70 min. The effluent perfusate was sampled in 5 min intervals and analysed for glucose and L-lactate. The AUC, areas under the curves (µmol g^{-1}) were obtained as described in Materials and Methods section. Data were expressed as mean ± SD of four individual liver perfusion experiments. $^{*}p < 0{\cdot}05$ versus normoglycaemic group

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 hypoglycaemic rats were investigated. For this purpose, the effect of saturating and physiological levels of L-alanine or L-glutamine on glucose production was compared. The physiological values of L-alanine and L-glutamine were obtained in a previous study.[16]

Statistical analysis

Data concerning glycaemia were analysed by analysis of variance (ANOVA) followed by Tukey's post-test. The results of liver perfusion experiments were analysed by the unpaired Student's *t*-test. Values are reported as mean ± SD. $p < 0{\cdot}05$ was accepted for all comparisons.

RESULTS

Livers of rats that received oral saline (normoglycaemic rats) or oral glibenclamide (hypoglycaemic rats) were infused with L-alanine (5 mM). Livers of hypoglycaemic rats showed lower ($p < 0{\cdot}05$) glucose (Figure 1A) and higher ($p < 0{\cdot}05$) pyruvate (Figure 1C) production than livers of normoglycaemic rats. The production of urea (Figure 1B) and L-lactate (Figure 1D) however remained unchanged.

In the next set of experiments livers from normoglycaemic and hypoglycaemic rats were infused with pyruvate (5 mM). Livers from hypoglycaemic rats showed lower ($p < 0{\cdot}05$) glucose (Figure 2A) and higher ($p < 0{\cdot}05$) L-lactate (Figure 2B) production than livers from normoglycaemic rats.

As showed by Figure 3 livers from normoglycaemic and hypoglycaemic rats were infused with L-glutamine (5 mM). Livers from hypoglycaemic rats showed lower ($p < 0{\cdot}05$) glucose production (Figure 3A) than livers from normoglycaemic rats. However, the urea production (Figure 3B) was not altered.

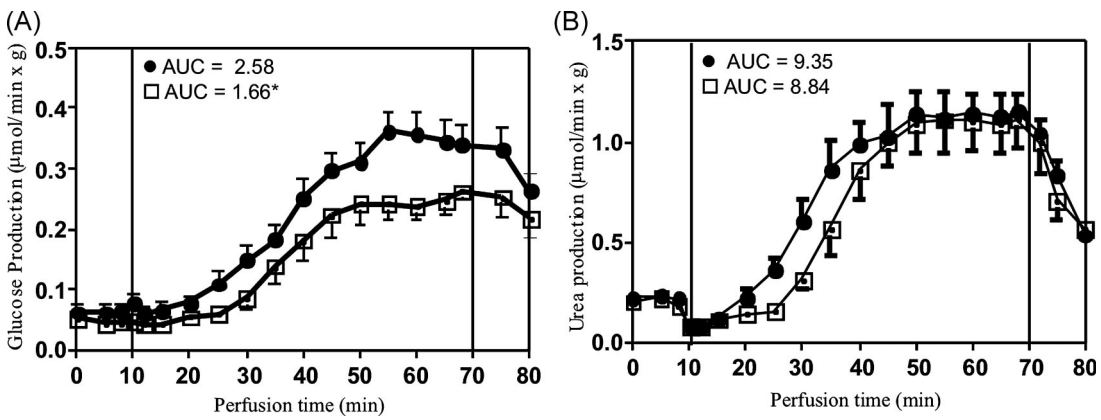

Figure 3. Glucose (A) and urea (B) production from L-glutamine (5 mM) in perfused livers of 24 h fasted rats that received oral (10 mg kg^{-1}) glibenclamide (hypoglycaemic, □) or saline (normoglycaemic, ●) 120 min before the liver perfusion experiments. L-glutamine was infused between 10 and 70 min. The effluent perfusate was sampled in 5 min intervals and analysed for glucose and urea. The AUC, areas under the curves (µmol g^{-1}) were obtained as described in Materials and Methods section. Data were expressed as mean ± SD of four individual liver perfusion experiments. $^{*}p < 0{\cdot}05$ versus normoglycaemic group

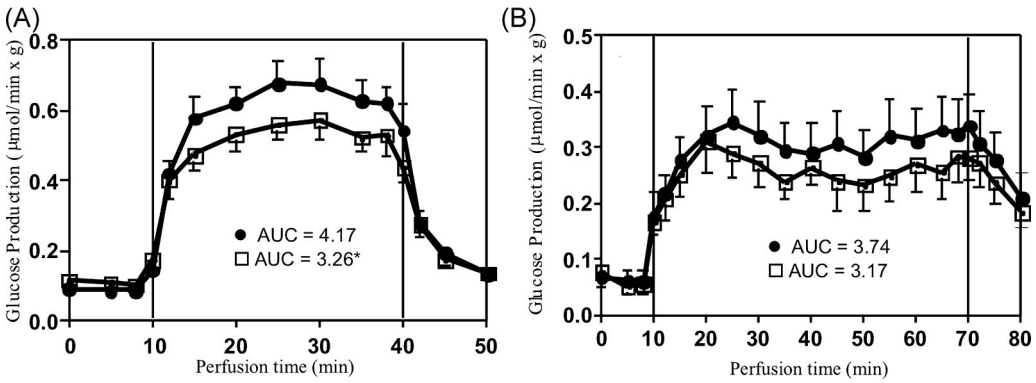

Figure 4. Glucose production from L-lactate 2 mM (A) and glycerol 2 mM (B) in perfused livers of 24 h fasted rats that received oral (10 mg kg^{-1}) glibenclamide (hypoglycaemic, □) or saline (normoglycaemic, ●) 120 min before the liver perfusion experiments. L-lactate or glycerol was between 10 and 70 min. The effluent perfusate was sampled in 5 min intervals and analysed for glucose. The AUC, areas under the curves (µmol g^{-1}) were obtained as described in Materials and Methods section. Data were expressed as mean ± SD of four individual liver perfusion experiments. $^{*}p < 0{\cdot}05$ versus normoglycaemic group

As showed by Figure 4 livers from normoglycaemic and hypoglycaemic rats were infused with L-lactate 2 mM (Figure 4A) or glycerol 2 mM (Figure 4B). Livers from hypoglycaemic rats showed lower ($p < 0{\cdot}05$) glucose production from L-lactate (Figure 4A) than livers from normoglycaemic rats. However, the glucose production from glycerol (Figure 4B) was similar for both groups.

Livers from hypoglycaemic rats infused with physiological (0·45 mM) or saturating (5 mM) concentration of L-alanine were compared. Livers infused with saturating concentration of L-alanine showed higher ($p < 0{\cdot}05$) glucose (Figure 5A) and urea (Figure 5B) production than livers infused with physiological concentration of L-alanine.

Finally, livers of hypoglycaemic rats infused with physiological (2·0 mM) and saturating (5 mM) levels of L-glutamine were compared. Livers infused with saturating levels of L-glutamine showed higher ($p < 0{\cdot}05$) glucose (Figure 6A) and urea (Figure 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. Pyruvate is carboxylated and leaves mitochondria as malate. In the cytosol, malate is converted to oxalacetate, then to phosphoenolpyruvate and after various steps including the microssomal glucose-6-phosphatase, to glucose that is released from the hepatocyte into the blood (Figure 7). Since this complex pathway depends on 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. Therefore, by using a saturating concentration of L-alanine we showed that the liver capacity to release glucose during SIH (Figure 1A) was decreased ($p < 0{\cdot}05$). This result could not be attributed to the decreased catabolism of L-alanine, since the urea production from

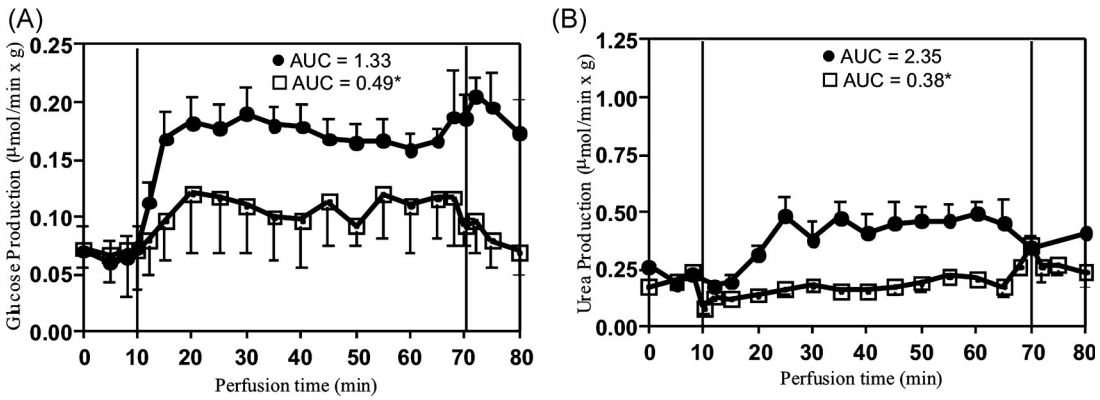

Figure 5. Glucose (A) and urea (B) production from L-alanine (5 mM, ●) and L-alanine (0·45 mM, □) in perfused livers of 24 h fasted rats that received oral (10 mg kg^{-1}) glibenclamide (hypoglycaemic) 120 min before the liver perfusion experiments. L-alanine was infused between 10 and 70 min. The effluent perfusate was sampled in 5 min intervals and analysed for glucose and urea. The AUC, areas under the curves (µmol g^{-1}) were obtained as described in Materials and Methods section. Data were expressed as mean ± SD of four individual liver perfusion experiments. $^{*}p < 0{\cdot}05$ versus physiological concentration, i.e. 0·45 mM

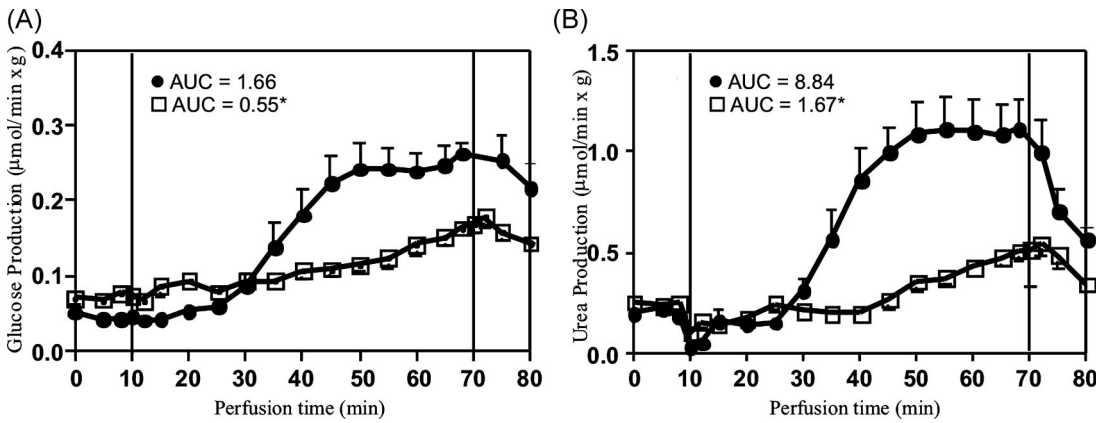

Figure 6. Glucose (A) and urea (B) production from L-glutamine (5 mM, ●) and L-glutamine (2·0 mM, □) in perfused livers of 24 h fasted rats that received oral (10 mg kg^{-1}) glibenclamide (hypoglycaemic) 120 min before the liver perfusion experiments. L-glutamine was infused between 10 and 70 min. The effluent perfusate was sampled in 5 min intervals and analysed for glucose and urea. The AUC, areas under the curves (μmol g^{-1}) were obtained as described in Materials and Methods section. Data were expressed as mean ± SD of four individual liver perfusion experiments. $^{*}p < 0{\cdot}05$ versus physiological concentration, i.e. 2·0 mM

this amino acid remained unchanged (Figure 1B). Moreover, considering that the pyruvate production (Figure 1C) from L-alanine in livers from SIH rats was increased ($p < 0{\cdot}05$), the possibility of a lower entrance of pyruvate in the gluconeogenic pathway must be considered.

In agreement with the proposition above, liver from SIH rats showed tendency of increased ($p > 0{\cdot}05$) production of L-lactate from L-alanine (Figure 1D) and increased ($p < 0{\cdot}05$) production of L-lactate from pyruvate (Figure 2B).

Consistent with the results of glucose production from L-alanine, liver from SIH rats also showed decreased ($p < 0{\cdot}05$) production of glucose from pyruvate (Figure 2A) and L-lactate (Figure 4A). It should be noticed that a direct effect of glibenclamide infused in the liver promoting inhibition of gluconeogenesis from L-lactate was previously demonstrated.[7]

Moreover, glucose production from L-glutamine, that enters in the gluconeogenic pathway after the pyruvate carboxylase (PC) step, was also decreased (Figure 3A). This result could not be attributed to the decreased catabolism of L-glutamine, since the urea production from this amino acid remained unchanged (Figure 3B). Nevertheless, the glucose production from glycerol, which enters in the

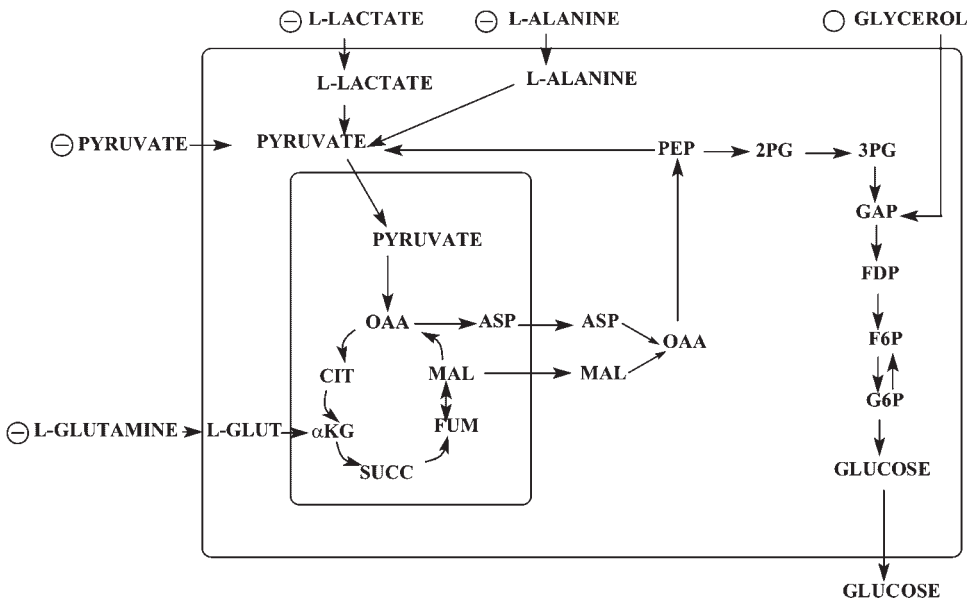

Figure 7. Gluconeogenesis in the hepatocyte. Plasma membrane is represented by the largest rectangle and mitochondria by the smallest rectangle. Decreased gluconeogenesis, ○ Maintained gluconeogenesis. Abbreviations: AcCoA, acetyl-CoA; ASP, aspartate; CIT, citrate; AG, fatty acid; FDP, fructose diphosphate; 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

gluconeogenesis after the mitochondrial step, was maintained (Figure 4B). In agreement with these results, an inhibition of the metabolic flux through the PC reaction by glibenclamide in rat liver was reported.[10]

The results suggest that the inhibition of gluconeogenesis promoted by glibenclamide overcome the intensification of liver gluconeogenesis in response to hypoglycaemia[2–5] mediated by insulin release. The mechanism by which glibenclamide promote inhibition of gluconeogenesis certainly involves uncouple oxidative phosphorylation.[18] In fact, early studies have found that sulphonylureas promoted inhibition of oxidative phosphorylation,[19] mitochondrial adenosine triphosphate activity,[20] and reduction of the membrane potential induced by succinate oxidation.[21] Thus, a reduced ATP/ADP in consequence of all these actions of glibenclamide help to explain the inhibition of liver gluconeogenesis.

Therefore, if these capabilities of the glibenclamide also occur *in vivo*, the result could be an intensification of the effectiveness of this drug as hypoglycaemic agent in the fasted state. However, this suggestion could not be expanded to the fed state, since in this condition glibenclamide stimulates glycogenolysis.[8]

In spite of the decreased liver capacity to release glucose during SIH, the possibility of the administration of gluconeogenic substrates during this condition must be considered. This affirmation is based on the fact that during SIH the hepatic glucose production from L-alanine (Figure 5A) and L-glutamine (Figure 6A) were lower ($p < 0{\cdot}05$), but maintained and influenced by the availability of these gluconeogenic substrates.

Finally, our results have great clinical interest because they help to understand the mechanism of hypoglycaemia induced by glibenclamibe, one of only two oral anti-diabetic agents available in the World Health Organization model list of essential medicines (the other being metformine). Moreover, 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.[22]

CONFLICT OF INTEREST

None known.

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