KATIA FIALHO DO NASCIMENTO

PAPEL DA DISPONIBILIDADE DE PRECURSORES GLICONEOGÊNICOS HEPÁTICOS EM DEFESA CONTRA A HIPOGLICEMIA INDUZIDA POR INSULINA EM RATOS

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Orientador: Prof. Dr. Roberto Barbosa Bazotte
Co–orientadora: Profa. Dra. Rosângela F. Garcia

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1. Pivotal role of hepatic gluconeogenic precursors availability in defense against hypoglycemia in rats. Artigo enviado para a revista *Life Sciences*.

PIVOTAL ROLE OF HEPATIC GLUCONEOGENIC PRECURSORS AVAILABILITY IN DEFENSE AGAINST HYPOGLYCEMIA IN RATS

Katia F. Nascimento[a], Rosângela F. Garcia[b], Gazola[b], V.A.F.G., Roberto B. Bazotte[c,*]

[a]CNPq Fellowship, State University of Maringá, Maringá, PR 87020-900, Brazil

[b]Department of Morphophysiological Sciences, State University of Maringá, Maringá, PR 87020-900, Brazil

[c]Department of Pharmacy and Pharmacology, State University of Maringá, Maringá, PR 87020-900, Brazil

*corresponding author:

Roberto Barbosa Bazotte

Department of Pharmacy and Pharmacology, State University of Maringá, Maringá, PR 87020-900, Brazil

Phone: +55-44-3261-4842

Fax: +55-44-3261-4999

e-mail: rbbazotte@uem.br

ABSTRACT

To understand better the mechanism of insulin induced hypoglycemia (IIH) we develop an experimental model in which hypoglycemia was obtained by an intraperitoneal injection of a pharmacological dose of regular insulin in 6 h food deprived rats. Using this experimental model we observed an increased capacity of the liver to produce glucose from saturating concentration of gluconeogenic substrates in livers of IIH rats. In light of these data we examined the role of the availability of liver glucose precursors to glycemia maintenance during IIH. For this purpose we compared the effect of basal and saturating concentration of gluconeogenic substrates in perfused livers *in situ* from IIH rats. In addition, the contribution of the liver availability of gluconeogenic substrates in defense against hypoglycemia was also investigated in *vivo* experiments (gavage). The results showed that livers from IIH rats which received saturating concentration of L-alanine, L-glutamine, L-lactate or glycerol had higher ($P < 0.05$) glucose, urea, pyruvate and L-lactate production than livers that received the basal blood concentration of these glucose precursors. In agreement, IIH rats that received oral L-alanine, L-glutamine or L-lactate showed higher ($P < 0.05$) glycemia 30 min after precursors administration. However, the effect of glycerol occurs immediately after its administration. It can be concluded that the availability of gluconeogenic substrates during IIH is crucial to hepatic gluconeogenesis and glycemia recovery.

KEY WORDS: hypoglycemia, hepatic gluconeogenesis, glycemia recovery, insulin, liver metabolism

INTRODUCTION

Over the past decade, the significance of intensive insulin therapy for chronic complications risk reductions has been convincingly demonstrated in a range of clinical studies (DCCT, 1993; UKPDS, 1998).

Today, no one question the value of intensive insulin therapy to protect against micro and macro vascular complications of diabetes. However, the rigorous glycemic control was associated with an increased incidence of insulin induced hypoglycemia (IIH) which is the major limitation for implementation of the intensive insulin treatment (Davis and Alonso, 2004).

To understand better the mechanisms of IIH we develop an experimental model in which hypoglycemia was obtained by an intraperitoneal injection of a pharmacological dose of regular insulin in 6 h food deprived rats (Souza et al., 1994a,b, 1996). Thus, by using this experimental model we observed an increased capacity of the liver to produce glucose from saturating concentration of gluconeogenic substrates in livers of IIH rats (Souza et al., 2001a,b). These results can be explained partly at least by the fact that during IIH, there is an increased release of glucagon (Dobbins et al. 1991; Chibber et al., 2000; Jiang et al., 2002), epinephrine (Bolli, 1998), grow hormone (Cryer, 1996) and cortisol (De Feo et al., 1989) that overcome the inhibitory effect of insulin on hepatic gluconeogenesis (Newsholme and Leech, 1983). However, this hormonal response was not enough to protect against IIH. A possible explanation for this discrepancy is the possibility that the counter regulatory hormones could not overcome the inhibitory effect of insulin on the release of gluconeogenic precursors from extra-hepatic tissues. In agreement

with this suggestion we observed decreased or maintained blood availability of L-alanine, L-glutamine, L-lactate and glycerol after insulin administration (Souza et al., 2001a,b; Garcia et al., in press; Gazola et al., 2007).

In light of these data it seemed important to examine the role of the availability of liver glucose precursors to glucose maintenance during IIH. For this purpose we compared the effect of saturating and physiological (basal blood values) concentration of gluconeogenic substrates in livers from IIH rats. In addition, the contribution of the effect of oral administration of gluconeogenic substrates in defense against hypoglycemia was investigated.

MATERIALS AND METHODS

Materials

Regular insulin (Neosulin®) was purchased from Biobras, Brazil. L-alanine and L-glutamine were obtained from ICN. All other reagents were of the highest purity obtainable.

Animals and treatment

Male Wistar rats weighing 180-220 g were maintained on standard rodent chow and water *ad libitum* before the initiation of the experimental procedures. On the day of the experiment, animals were food deprived from 8:00 a.m. The experiments were performed with 6 h food deprived rats. The manipulation of the animals followed the Brazilian law on the protection of animals.

Previous experiments (Souza et al., 1994a,b) from our laboratory were done to characterize blood glucose and insulin after an intraperitoneal injection (1 U/kg) of regular insulin. Blood was obtained from rats killed by decapitation. The values of

insulinemia after insulin injection at 0, 30 and 60 min (n = 7) were 27.0 ± 5.3 µU/ml, 227.0 ± 39.0 µU/ml and 136.0 ± 26.4 µU/ml, respectively. The values of glycemia after insulin injection at 0, 30 and 60 min (n = 7) were 104.4 ± 2.16 mg/dl, 50.4 ± 4.68 mg/dl, 45.0 ± 8.1 mg/dl, respectively. Additionally the blood concentration of L-alanine, L-glutamine, L-lactate and glycerol 60 min after insulin injection were 0.21 mM; 1.05 mM; 1.16 mM; 0.045 mM respectively.

Liver perfusion experiments

IIH rats were anesthetized by an intraperitoneal injection of sodium thiopental (40 mg/Kg) and submitted to laparotomy. The experimental protocol described in the Fig. 1 was employed: after a short perfusion with Krebs/Henseleit-bicarbonate (KH) period (0-10 min), glycerol was infused between 10-70 min, followed by an additional perfusion period with KH (70-80 min) to allow a return to basal glucose production. Samples of the effluent perfusion fluid were collected at 2 min intervals and analyzed for glucose (Bergmeyer and Bernt, 1974). The differences in the glucose production during (10-70 min) and before (0-10 min) the infusion of glycerol allowed calculation the areas under the curves (AUC), expressed as µmol/g. In part of the liver perfusion experiments L-lactate (Gutmann and Wahlefeld, 1974), pyruvate (Czok and Lamprecht, 1974) and urea (Gutmann and Bergmeyer, 1974) production were measured.

Thus, the AUCs shown in fig. 2-4 were obtained from similar experiments to that illustrated in Fig. 1.

To verify the role of the gluconeogenic substrates availability to promote glucose production in livers of IIH rats, saturating (5 mM, 5 mM, 2 mM and 2mM to L-alanine, L-glutamine, L-lactate and glycerol, respectively) and basal (0.21 mM; 1.05

mM; 1.16 mM; 0.045 mM to L-alanine, L-glutamine, L-lactate and glycerol, respectively) concentration of each substrate were compared. The saturating and basal values of L-alanine, L-glutamine, L-lactate and glycerol were obtained in a previous study (Souza, 1999).

At this point it must be emphasized that the saturating values represents the concentration of the substrate in which the maximal hepatic glucose production was obtained. Moreover, the basal values of L-alanine, L-glutamine, L-lactate and glycerol represent the blood levels of each substrate 60 min after insulin administration.

Oral administration of hepatic glucose precursors

IIH was well-established 30 min after insulin injection and so this period of time was selected for oral administration of gluconeogenic substrates. Thus, L-alanine (100 mg/kg), L-glutamine (100 mg/kg), L-lactate (100 mg/kg) or glycerol (100 mg/kg) were orally administered (gavage) 30 min after insulin injection. Moreover, an additional IIH group that received an equal volume of saline was included (SAL group). Blood samples to evaluate glycemia were collected (by decapitation) 30 min later, i.e., 60 min after insulin injection. The dose utilized of L-alanine, L-glutamine, L-lactate and glycerol were determined in a previous study (Souza, 1999). Moreover, to glycerol an additional experiment was done in which blood samples to measure glycemia were collected by decapitation 2, 5, 8, 10, 15, 20, 25 and 30 min after glycerol administration, i.e., 32, 35, 38, 40, 45, 50, 55 and 60 min after insulin administration.

Statistical analysis

Results are reported as means ± SD. The program GraphPad Prism (version 4.0) was used to calculate the AUC. Data were analyzed statistically by the unpaired Student's t-test. $P < 0.05$ was accepted for all comparisons.

RESULTS

As shown by Fig. 2, livers from IIH rats which received saturating concentration of L-alanine, L-glutamine, L-lactate or glycerol showed higher ($P < 0.05$) glucose production than livers that received the basal blood concentration of these glucose precursors.

Livers of IIH rats that received saturating concentration of L-alanine or L-glutamine also shown higher ($P < 0.05$) urea production than livers that received basal concentration of these glucose precursors (Fig. 3). In agreement, the pyruvate production from saturating levels of L-alanine or L-lactate was higher ($P < 0.05$) than that obtained in livers of IIH rats that received basal concentration of these glucose precursors (Fig 4). Moreover, the L-lactate production was higher ($P < 0.05$) in livers of IIH rats that received saturating levels of L-alanine (not shown).

Finally, IIH rats that received oral L-alanine, L-glutamine or L-lactate showed higher ($P < 0.05$) glycemia 30 min after the administration of these substances (Fig. 5). However, to glycerol higher glycemia ($P < 0.05$) was observed until 8 min after its administration (Fig. 6).

DISCUSSION

Perfused livers of fasted rats produce negligible amounts of glucose in the absence of glucose precursors. But, the infusion of a saturating concentration of gluconeogenic substrate increases the rate of glucose production (Fig. 1).

Since gluconeogenesis depends of the availability of gluconeogenic substrates (Pilks and Granner, 1992) and the fact that during IIH the concentration of glucose precursor was decreased or maintained (Souza et al, 2001a,b; Garcia et al., in press; Gazola et al., 2007). We investigated the role of the availability of gluconeogenic substrates to the hepatic gluconeogenesis by comparing the effect of basal and saturating concentration of L-alanine, L-lactate, L-glutamine or glycerol in livers of IIH rats.

Thus, by comparing basal and saturating concentration of L-alanine, L-lactate, L-glutamine or glycerol we observed that during IIH the ability of the liver to produce glucose depends of the availability of gluconeogenic substrates. The conclusion is based in the fact that livers of IIH rats shown higher ($P < 0.05$) glucose production from saturating levels of these substances (Fig. 2).

The higher hepatic glucose production from saturating concentration of L-alanine and L-glutamine was probably the result of the increased catabolism of these amino acids, inferred by the higher ($P < 0.05$) urea production (Fig. 3), which favored the generation of gluconeogenic intermediaries to the liver (Newsholme and Leech, 1983). In line with this observation livers from IIH rats which received saturating concentration of L-alanine showed higher ($P < 0.05$) hepatic production of pyruvate (Fig. 4) and L-lactate (not shown). In agreement, livers from IIH rats which received saturating concentration of L- lactate showed higher ($P < 0.05$) hepatic production of

pyruvate (Fig. 4). The increased production of L-lactate and pyruvate favored gluconeogenesis and helps to explain the largest hepatic glucose production showed by livers which received saturating concentration of L- alanine or L-lactate.

Since our results employing *in situ* perfused livers showed that the availability of gluconeogenic substrates is crucial to hepatic glucose production we decided to verify whether the oral administration of L-alanine, L-lactate, L-glutamine or glycerol (each 100 mg/kg) could promote glucose recovery.

As illustrated by Fig. 5, rats that received L-alanine, L-lactate or L-glutamine 30 min after insulin administration showed higher ($P < 0.05$) glycemia than rats which received saline 30 min later. Moreover, the absence of glycemia recovery after glycerol administration, in contrast with its better performance as gluconeogenic substrate (Fig. 2) was unexpectedly. To verify the possibility that the effect of glycerol on glycemia occurs before 30 min after its administration, an additional experiment was executed. The results, showed that in contrast with L-alanine, L-lactate or L-glutamine, the effect of oral glycerol on glycemia occur until 8 min after its administration (Fig. 6). A possible reason for glycemia recovery from glycerol occurs immediately after its administration may be the fact that glycerol gets portal vein faster than other gluconeogenic substrates (Kin-ya et al., 2006; Hiroaki et al., 2003).

Thus, we conclude that the administration of gluconeogenic precursors during IIH promoted glucose recovery due to the increased blood availability of gluconeogenic substrates. Finally, our previous investigations (Souza et al., 2001 a,b) and the considerations herein discussed, open the possibility to the administration of glucose precursors in the treatment of IIH, particularly glycerol which promoted effect against hypoglycemia at least 2 min after its administration

(Fig. 6). However, additional experimental and future clinical investigations will be necessary to confirm this suggestion.

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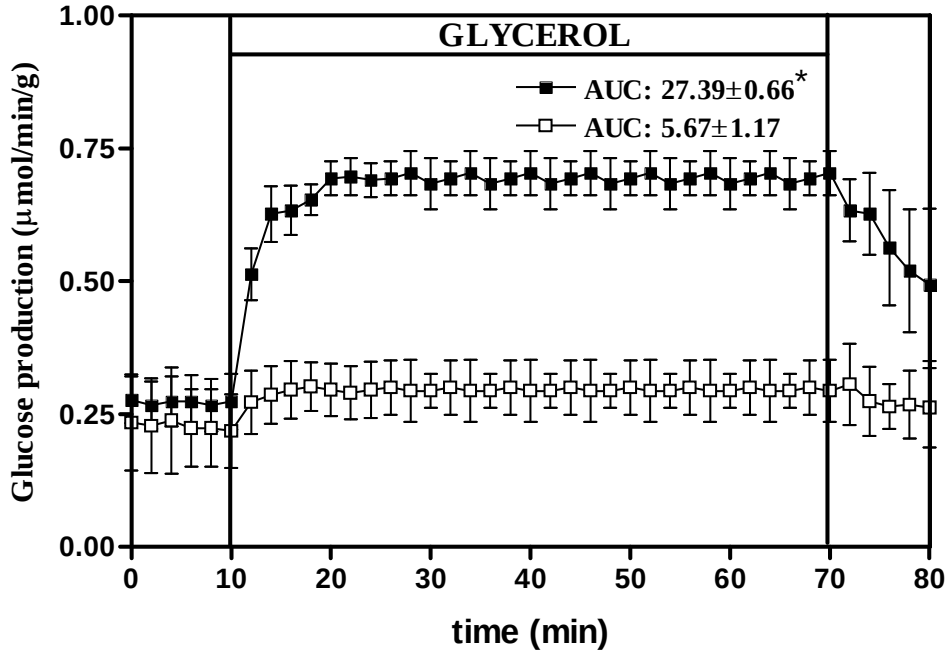

Fig.1. Demonstrative experiments of glucose production from basal (☐) and saturating (■) concentration of glycerol in perfused livers of hypoglycemic 6 h food deprived rats that received an intraperitoneal injection (1 U/kg) of insulin 60 min before the experiment. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose. The data are expressed as the mean ± SD (n= 6-8). AUC - area under the curves (µmol/g). Asterisks indicate a significante difference, *P < 0.05 *vs* basal level.

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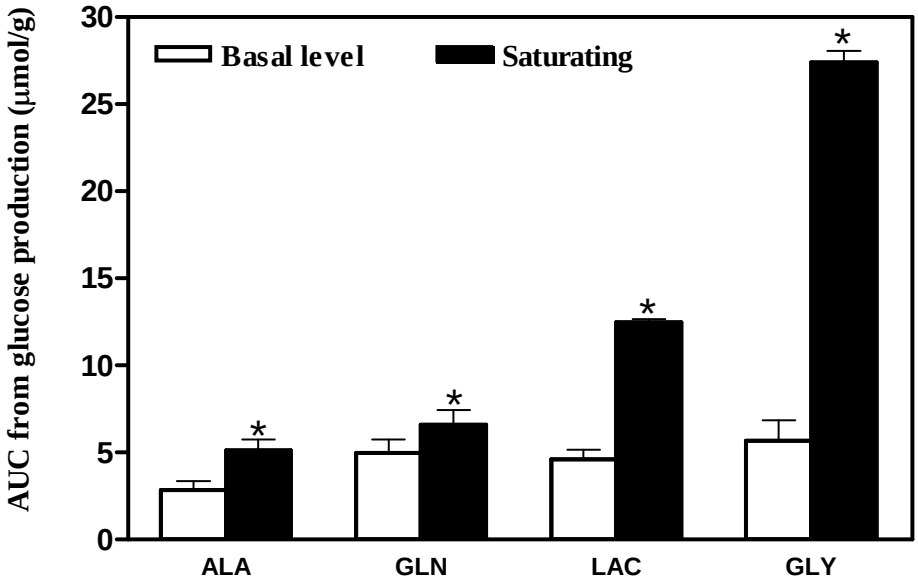

Fig.2. Glucose production from basal and saturating concentration of L-alanine (ALA), L-glutamine (GLN), L-lactate (LAC) or glycerol (GLY) in perfused livers of hypoglycemic 6 h food deprived rats. Data were expressed as means ± SD (n= 6-8). AUC= area under the curves. Asterisks indicate a significante difference, *P < 0.05 vs basal level.

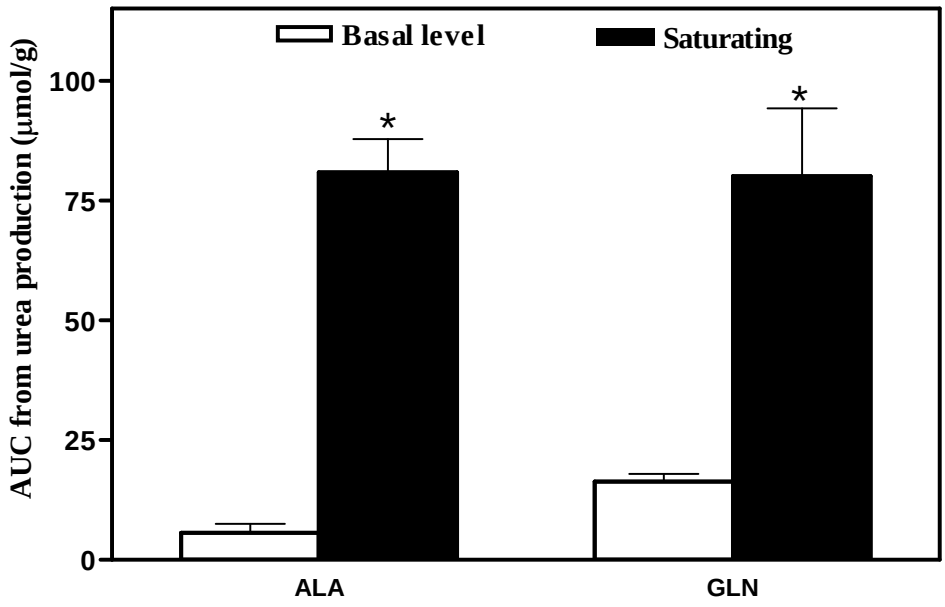

Fig.3. Urea production from basal and saturating concentration of L-alanine (ALA) or L-glutamine (GLN) in perfused livers of hypoglycemic 6 h food deprived rats. Data were expressed as means ± SD (n= 6-8). AUC= area under the curves. Asterisks indicate a significante difference, *P< 0.05 *vs* basal level.

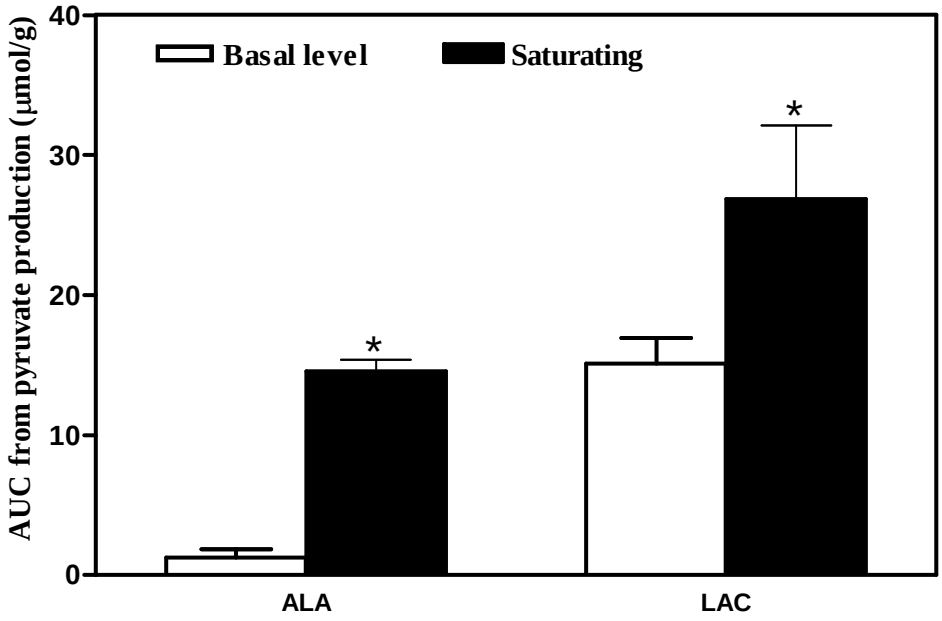

Fig.4. Pyruvate production from basal and saturating concentration of L- alanine (ALA) or L- lactate (LAC) in perfused livers of hypoglycemic 6 h food deprived rats. Data were expressed as means ± SD (n= 6-8). AUC= area under the curves. Asterisks indicate a significante difference, *P < 0.05 *vs* basal level.

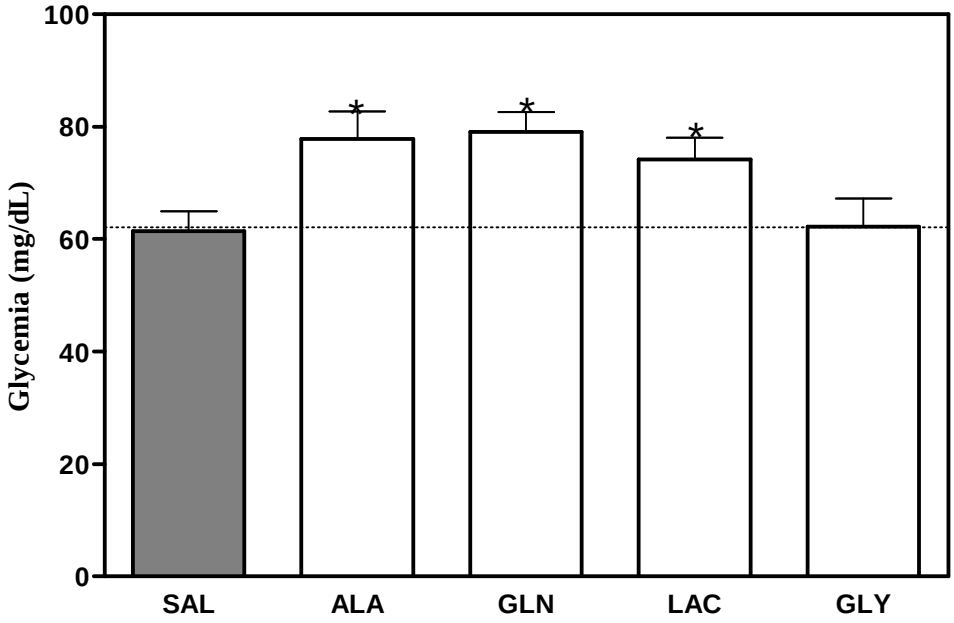

Fig.5. Effect of the oral administration of saline (SAL), 100 mg/kg L-alanine (ALA), 100 mg/kg L-glutamine (GLN), 100 mg/Kg L-lactate (LAC), or 100 mg/kg glycerol (GLY) on glycemia in hypoglycemic 6 h food deprived rats. The gluconeogenic substrates were orally administered 30 min after insulin injection. Glycemia was measured 30 min later, i.e., 60 min after insulin injection. Data were expressed as means ± SD (n= 6-8). Asterisks indicate a significante difference, *P < 0.05 *vs* SAL group.

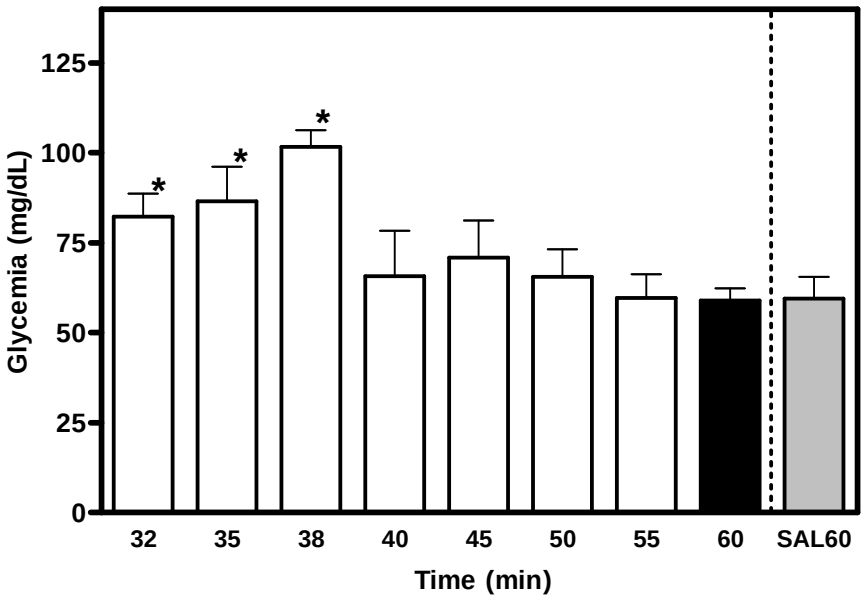

Fig.6. Effect of oral administration of glycerol (100 mg/kg) on glycemia in hypoglycemic 6 h food deprived rats. Glycemia was measured 32, 35, 38, 40, 45, 50, 55 and 60 min (■) after insulin injection, i.e., 2, 5, 8, 10, 15, 20, 25 and 30 min after glycerol administration. Key: SAL 60 represents the group in which glycemia were measured 60 min after saline injection. Data were expressed as means ± SD (n= 6-8). Asterisks indicate a significante difference, *P < 0.05 *vs SAL* 60 group.

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