

Oral lactate intensifies insulin toxicity during severe insulin-induced hypoglycemia in mice

Vanessa Rodrigues Vilela^{1#}, Marina Masetto Antunes^{1#}, Vilma Aparecida Ferreira Godoi², Patricia Batista Travassos¹, Helenir Medri de Souza³, Roberto Barbosa Bazotte^{1*}

¹Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, Paraná, Brazil, ²Department of Physiological Sciences, State University of Maringá, Maringá, Paraná, Brazil, ³Department of Physiological Sciences, State University of Londrina, Londrina, Paraná, Brazil

We investigated whether oral lactate could prevent seizures and deaths in mice with severe hypoglycemia induced by a high dose of insulin. For this purpose, mice were fasted for 15 h and then given an intraperitoneal injection of regular insulin (5.0 U/kg or 10.0 U/kg). Immediately after insulin injection, the mice received an oral dose of saline (control), glucose (5.5 mmol/kg), or lactate (18.0 mmol/kg). Glucose and lactate levels were measured in the blood and brain before and after the seizures began. Glucose and lactate delayed ($p < 0.05$) the onset of seizures associated with severe insulin-induced hypoglycemia. Elevated ($p < 0.05$) brain levels of lactate were associated with an absence of seizures in mice that received glucose or lactate, suggesting that lactate could prevent convulsions associated with severe insulin-induced hypoglycemia. However, the same oral dose of lactate that delayed the onset of convulsions also increased the mortality rate. In contrast, diazepam (3.0 mg/kg) prevented seizures and markedly decreased the frequency of death during severe insulin-induced hypoglycemia. The results demonstrated that in contrast to oral glucose, oral lactate intensifies insulin toxicity.

Keywords: Lactatemia. Oral glucose. Diazepam. Seizure. Anticonvulsant effect.

INTRODUCTION

Insulin-induced hypoglycemia, the major acute adverse effect of insulin therapy (Sanches *et al.*, 2013; Vilela *et al.*, 2014), is clinically relevant because glucose is the main fuel for the brain (Bazzigaluppi *et al.*, 2017).

Because glucose administration has an evanescent effect in treating hypoglycemia, diabetes patients must be advised to eat regular meals to prevent a recurrence of insulin-induced hypoglycemia. However, during sleep, this method is not adequate to treat nocturnal hypoglycemia. In fact, nocturnal hypoglycemia associated with “dead in bed syndrome” is responsible for 5-6% of deaths among patients with type 1 diabetes (Weston, 2012).

Therefore, it is necessary replace glucose with substances that are more potent and have prolonged effects.

In this regard, the effects of glucose have previously been compared with those of glutamine, alanine, lactate, and pyruvate (Galende *et al.*, 2009; Hartman *et al.*, 2010; Nunes Santiago *et al.*, 2013; Rodrigues *et al.*, 2011).

Because glucose inhibits liver gluconeogenesis and does not alleviate hypoglycemia induced by insulin administration (Galende *et al.*, 2009), liver glucose continues to be produced from lactate during periods of hypoglycemia (Hartman *et al.*, 2010; Rodrigues *et al.*, 2011). Moreover, lactate has been proposed as an alternative brain energy fuel (Pellerin, Magistretti, 1994, Wyss *et al.*, 2011). In fact, the lactate produced from glucose in astrocytes is released and used by neurons. Thus, the concept of glucose being the main brain fuel remains unchanged; however, the current idea is that lactate produced from glucose, and not glucose *per se*, is the main energy fuel for neurons (Pellerin, Magistretti, 1994; Pellerin, Magistretti, 2012).

When coupled with results from our previous study (Hartman *et al.*, 2010; Rodrigues *et al.*, 2011), this new concept concerning the role of lactate as a brain fuel

*Correspondence: R. B. Bazotte. Departamento de Farmacologia e Terapêutica, Universidade Estadual de Maringá. Avenida Colombo 5790 - Bloco K68 – sala 101, CEP: 87020-900 - Maringá – PR – Brasil. Tel.: +55-44-3011-5161; Fax: +55-44-3011-5050. E-mail: rbbazotte@gmail.com

This authors equally contributed to the present work.

(Pellerin, Magistretti, 2012; Proia *et al.*, 2016; Smith *et al.*, 2003), suggests the possibility of using lactate rather than glucose to treat insulin induced hypoglycemia (Chan *et al.*, 2013; De Feyter *et al.*, 2013; Herzog *et al.*, 2013; Oldenbeuving *et al.*, 2014; Rooijackers *et al.*, 2016).

Here, we evaluate the effect of oral lactate on the occurrence of seizures and deaths among mice that received high doses of insulin. To complement this investigation, the blood and brain levels of glucose and lactate, and the ability of diazepam to prevent seizures and deaths were also evaluated.

MATERIAL AND METHODS

Animals

The study protocol was approved by the Animal Ethics Committee (079-PRO 051.2011) of the State University of Maringá. All experimental procedures were performed in accordance with the Brazilian Directives on Research Animal Protection and Experimentation (11.794/2008), and reported in accordance with ARRIVE (Animal Research: Reporting *in Vivo* Experiments).

Mice (6-weeks of age) were fasted for 15 h, and then given a single intraperitoneal (*ip*) injection of regular insulin (Humulin®). Immediately after insulin injection, the mice were randomly selected to receive an oral gavage dose of glucose (5.5 mmol/kg), or lactate (18.0 mmol/kg), or a single *ip* dose of diazepam (3.0 mg/kg).

The doses of insulin (Deprez-Poulain *et al.*,

2015; Wang, Liao, 2012), glucose (Guo *et al.*, 2015), lactate (Thurston, Hauhart, Schiro, 1983) and diazepam (Keshavars, Showraki, Emamghoreishi, 2013) were selected based on doses used in previous studies.

Dose response curve and seizures

Mice were divided into 8 groups ($n = 3-4$ per group) which were given increasing *ip* doses of insulin: (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, or 10.0 U/kg). Blood samples were collected from the tail at 0, 30, 60, 90, and 120 min after insulin injection, as previously described (Marques *et al.*, 2016). Glycemia (mmol/L) was evaluated with a home glucometer (Optium Xceed®, Abbott Laboratories, Chicago, IL, USA), which is known to show good accuracy compared with the gold standard method used to measure plasma glucose (Robinson, Sharp, 2012).

The results of these experiments are summarized in the Figure 1.

Because seizures were observed only after insulin was administered at a dose of 5.0 U/kg or 10.0 U/kg (Figure 1), those doses were chosen to evaluate the effect of oral glucose or lactate administration on seizures and deaths.

The effects of oral glucose or lactate on seizures and deaths among hypoglycemic mice

The effects of oral glucose (5.5 mmol/kg) or oral lactate (18.0 mmol/kg) on seizures and deaths among

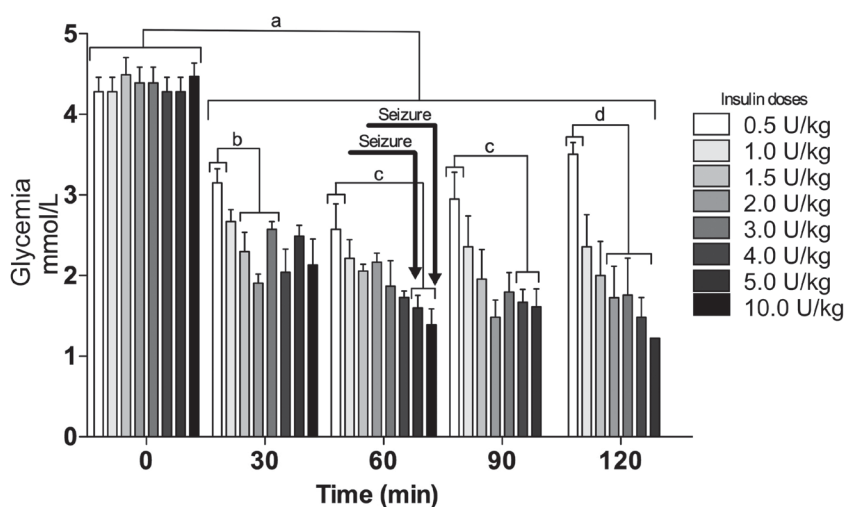


FIGURE 1 - Dose response curve and seizures. Blood glucose levels at 0, 30, 60, 90, and 120 min after an intraperitoneal injection (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 or 10.0 U/Kg) of insulin into 15 h fasted mice. The mice were sacrificed when a seizure started. Results represent the mean \pm standard error ($n = 3-4$). Data were analyzed using the Newman-Keuls multiple comparisons test. ^a $p < 0.05$ as compared to time 0; ^b $p < 0.05$ as compared to the dose (1.5 U/kg, 2.0 U/kg or 3.0 U/kg); ^c $p < 0.05$ as compared to the dose (5.0 U/kg or 10.0 U/kg); ^d $p < 0.05$ as compared to the dose (2.0 U/kg, 3.0 U/kg or 4.0 U/kg).

mice that received *ip* insulin (5.0 U/kg or 10.0 U/kg) were evaluated.

Thirty mice that received a 5.0 U/kg dose of insulin and thirty mice that received a 10.0 U/kg dose of insulin were divided into the following 3 groups (n = 10 mice per group): insulin + saline, insulin + lactate, and insulin + glucose. Saline, lactate or glucose was administered immediately after the insulin injection. The mice were then observed for a period of 300 min, which started immediately after an *ip* dose of insulin followed by an oral dose of saline, lactate or glucose.

To evaluate seizures after insulin injection, each group of mice (10 mice per cage) was kept in a silent room. While in the room, data for the following parameters was recorded for each group of mice: the of onset of the first seizure; time between seizures; number of seizures; death rate; time of death. As previously described by Zhao *et al.* (2003), seizure was defined as when the mouse showed rhythmic jerks (clonic) and continuous tension or contraction of muscles (tonic).

Blood and brain levels of glucose and lactate

The blood and brain levels of glucose and lactate in mice that received *ip* insulin (10.0 U/kg) + oral saline (Ins group), *ip* insulin (10.0 U/kg) + oral lactate (Ins + Lac group) or *ip* insulin (10.0 U/kg) + oral glucose (Ins + Glc group) were quantified. A normoglycemic Control group that received *ip* saline and oral saline (Control group) was also included.

All mice (n = 6 per group) were sacrificed by decapitation. This method ensures a quick blood and brain collection without interference from anesthetics in the brain.

After decapitation, the blood was collected, centrifuged (10 min at 5 °C), and the plasma was kept on ice for subsequent evaluations of blood lactate and glucose levels. Lactate concentrations were measured using enzymatic techniques (Gutmann, Wahlefeld, 1974). Glucose was measured by using commercial kits.

For evaluations of brain lactate and glucose levels, the brains were quickly removed and stored in liquid nitrogen for several minutes. The brains were then homogenized (20 seconds) with perchloric acid containing EDTA (1 mM) in a van Potter-Elvehjem homogenizer; after which, the homogenates were placed in an ice bath and centrifuged at 10000 g (for 20 min at 5 °C.) The supernatant fractions were neutralized with KOH (5 mmol) containing 50 mmol triethanolamine, and then maintained on ice for 15 min before being centrifuged again (Kepler, Decker, 1974). After centrifugation, the supernatant lactate and glucose levels were measured

using enzymatic techniques (Gutmann, Wahlefeld, 1974) and commercial kits, respectively.

The following studies were performed as two sets of experiments.

In one set of experiments, the mice (n = 6 per group) were sacrificed when a seizure started in the Ins group but not in the other groups. This experimental approach allowed us to compare mice with a seizure (Ins group) and without a seizure. In the other set of experiments, each mouse was sacrificed when it began to convulse (n = 4 per group). This experimental approach allowed for comparisons of all mice which experienced a seizure.

The effects of diazepam on seizures and deaths among mice that received *ip* insulin

Mice that received *ip* insulin (10.0 U/kg) + *ip* saline (n = 9 per group) were compared with mice that received *ip* insulin (10.0 U/kg per group) + *ip* diazepam (3.0 mg/kg) (n = 10 per group). The 300 min observation period started immediately after injection of either *ip* insulin + *ip* saline or *ip* insulin + *ip* diazepam.

Statistical analyses

All data were analyzed by ANOVA (Newman-Keuls post hoc test) using Graph-Pad Prism Version 5.0 software. Results are reported as the mean ± standard error (SE) of the mean. P-values < 0.05 were considered statistically significant.

RESULTS

As shown in Table I, 90%, 100%, and 80% of mice that received *ip* insulin (5.0 U/kg) plus oral saline, *ip* insulin (5.0 U/kg) plus oral lactate or *ip* insulin (5.0 U/kg) plus oral glucose, respectively, had seizures.

The number of seizures in the group that received *ip* insulin (5.0 U/kg) plus oral glucose was higher (p < 0.05) than those in the other groups. Moreover, the group that received *ip* insulin (5.0 U/kg) plus oral lactate had a higher death rate (Table I).

Seizure rate, onset of first seizure, number of seizures, time between seizures, death rate, and time of death during the 300 min observation period after *ip* insulin injection. Oral saline, lactate or glucose was administered immediately after insulin injection. Data are reported as the mean ± standard error or a proportion (n/n). Data were analyzed using the Newman-Keuls multiple comparisons test. ^ap < 0.05 as compared to the Insulin + saline group; ^bp < 0.05 as compared to the Insulin + lactate group.

TABLE I - Effect of oral glucose (5.5 mmol/kg) or lactate (18.0 mmol/kg) on seizures and deaths among hypoglycemic mice that received intraperitoneal insulin (5.0 U/kg)

	Insulin + Saline	Insulin + Lactate	Insulin + Glucose
Seizure rate	9/10	10/10	8/10
Onset of first seizure (min)	71.7 ± 11.7 n = 9	100.4 ± 9.0 n = 10	83.3 ± 6.3 n = 8
Number of seizures	4.8 ± 1.5 n = 9	2.5 ± 0.8 n = 10	8.5 ± 1.1 ^{a,b} n = 8
Time between the seizures (min)	14.5 ± 1.3 n = 5	17.5 ± 6.9 n = 6	21.4 ± 11.1 n = 8
Death rate	1/10	7/10	1/10
Time of death (min)	192.8 n = 1	139.0 ± 10.0 n = 7	170.0 n = 1

As shown in Table II, all mice that received *ip* insulin (10.0 U/kg) had seizures.

Furthermore, the seizures started earlier ($p < 0.05$) in the group that received *ip* insulin (10.0 U/kg) + oral saline than in the other groups. Moreover, the group that received *ip* insulin (10.0 U/kg) + oral lactate had a higher death rate.

Seizure rate, onset of the first seizure, number of seizures, time between seizures, death rate, and time of death during the 300 min observation period following *ip* administration of insulin. Oral saline, lactate or glucose was administered immediately after insulin injection. Results are reported as the mean ± standard error or a proportion (n/n). Data were analyzed using the Newman-Keuls multiple comparisons test. ^a $p < 0.05$ as compared to the Insulin + saline group.

Because all mice given 10.0 U/kg of insulin had seizures, that dose was chosen to evaluate the glucose and lactate levels in the blood and brain.

The data shown in Figure 2A-D, compare the blood

and brain glucose and lactate levels of mice with a seizure (Ins group) and without a seizure (Ins + Glc group and Ins + Lac group).

The glycemia values of all groups that received *ip* insulin were lower ($p < 0.05$) than those in the Control group (Figure 2A). Additionally, the Ins + Glc group had higher ($p < 0.05$) glycemia values than the Ins group (Figure 2A). In contrast, the brain glucose levels were similar in all groups (Figure 2B).

The lactatemia values were greater ($p < 0.05$) in the Ins + Lac group than in the other groups. In contrast, the Control, Ins, and, Ins + Glc groups had similar blood lactate levels (Figure 2C).

Moreover, the Ins group had lower ($p < 0.05$) brain lactate levels than the other groups (Figure 2D).

The results presented in Figure 2E-H compare the blood and brain glucose and lactate levels of all animals after seizure onset in each animal; thus the levels were measured at variable intervals after receiving insulin.

TABLE II - Effect of oral glucose (5.5 mmol/kg) or lactate (18.0 mmol/kg) on seizures and deaths among hypoglycemic mice that received intraperitoneal insulin (10.0 U/kg)

	Insulin + Saline	Insulin + Lactate	Insulin + Glucose
Seizure rate	10/10	10/10	10/10
Onset of first seizure (min)	59.9 ± 8.1 n = 10	109.9 ± 5.6 ^a n = 10	127.8 ± 8.2 ^a n = 10
Number of seizures	7.8 ± 1.1 n = 10	2.8 ± 0.3 ^a n = 10	3.7 ± 0.6 ^a n = 10
Time between the seizures (min)	21.5 ± 5.9 n = 10	33.3 ± 8.2 n = 10	18.4 ± 3.3 n = 10
Death rate	3/10	8/10	2/10
Time of death (min)	191.1 ± 47.5 n = 3	186.6 ± 18.3 n = 8	150.9 ± 19.2 n = 2

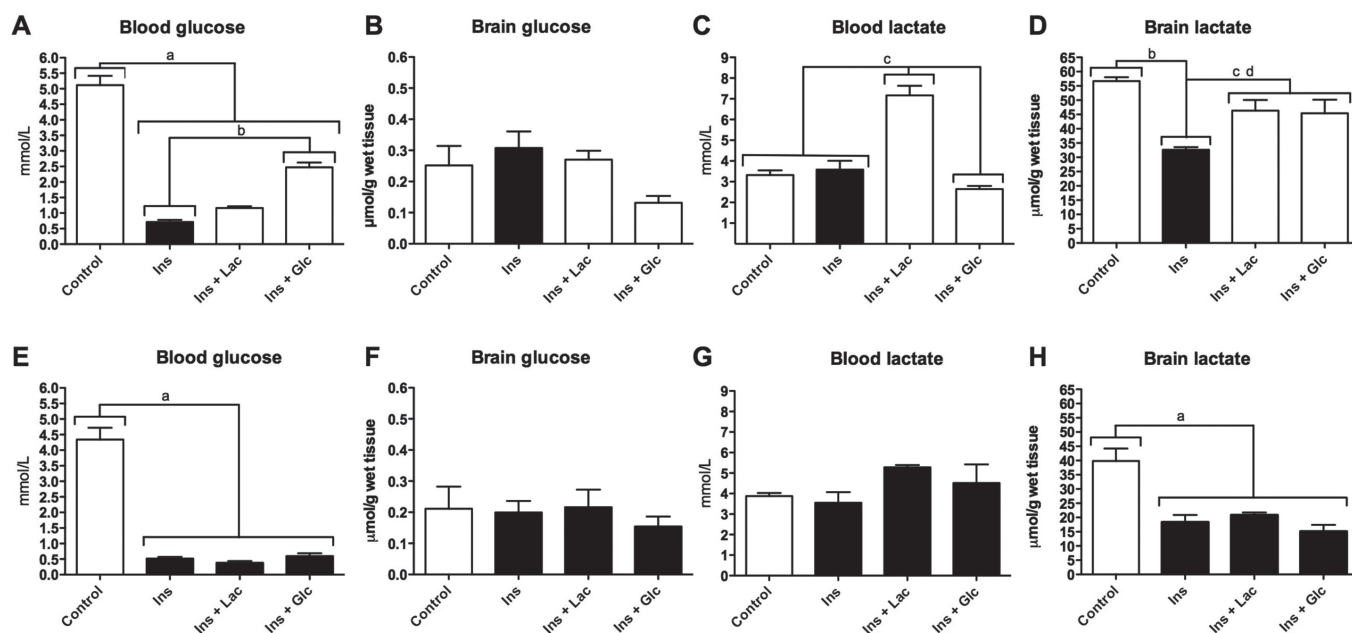


FIGURE 2 - Blood and brain levels of glucose and lactate in mice with (■) and without (□) seizures. The Control group received intraperitoneal (*ip*) saline + oral saline. The experimental groups received *ip* insulin + oral saline (Ins group), *ip* insulin + oral lactate (Ins and Lac group) or *ip* insulin + oral glucose (Ins and Glc group). Oral saline, lactate (18.0 mmol/kg) or glucose (5.5 mmol/kg) was administered immediately after *ip* saline (Control group) or insulin (10.0 U/kg). In one set of experiments (A, B, C, D; n = 6), the mice were sacrificed when the first seizure started in the Ins group, and while the other groups did not have seizures. In the other set of experiments, each mouse was sacrificed when it began seizing (E, F, G, H; n = 4). Data are reported as the mean ± standard error. Data were analyzed using the Newman-Keuls multiple comparison test, ^ap < 0.05 as compared to the Control group; ^bp < 0.05 as compared to the Ins group; ^cp < 0.05 as compared to the Ins + Lac group; ^dp < 0.05 as compared to the Ins + Glc group.

The glycemia (Figure 2E) and brain lactate levels (Figure 2H) were lower (p < 0.05) in all groups that received *ip* insulin than in the Control group. However, the Ins, Ins + Lac, and Ins + Glc groups showed no difference in their glycemia (Figure 2E), blood lactate (Figure 2G), brain glucose (Figure 2F) and brain lactate (Figure 2H) levels.

The numbers of seizures and deaths among mice that received *ip* insulin (10.0 U/kg) and mice that received *ip*

insulin (10.0 U/kg) plus *ip* diazepam (3.0 mg/kg) were compared. As shown in Table III the *ip* administration of diazepam prevented seizures and decreased the death rate among mice that received *ip* insulin (10.0 U/kg).

DISCUSSION

Nocturnal hypoglycemia, especially common in type 1 diabetic patients, is a danger for any patient being treated

TABLE III - Effect of intraperitoneal (*ip*) diazepam (3.0 mg/kg) on seizures and the death rate among mice that received *ip* insulin (10.0 U/kg)

	Seizure rate	Onset of first seizure (min)	Number of seizures	Time between the seizures (min)	Death Rate	Time of death (min)
Insulin + Saline	9/9	67.9 ± 8.9 n = 9	7.4 ± 1.2 n = 9	19.5 ± 4.4 n = 9	5/9	174.9 ± 27.6 n = 5
Insulin + Diazepam	0/10	NA n = 10	NA n = 10	NA n = 10	1/10	172.6 ± 0 n = 1

Seizure rate, onset of first seizure, number of seizures, time between seizures, death rate, and time of death during the 300 min observation period following *ip* administration of insulin and either *ip* saline or *ip* diazepam. Results are reported as the mean ± standard error or a proportion, (n/n). NA: not applicable

by insulin because the patient is not awake and cannot take appropriate measures or receive help.

In view of the vulnerability of the brain and the absence of suitable antidotes for nocturnal hypoglycemia, the possibility of using lactate to treat or prevent prolonged hypoglycemia must be considered.

The main reason for this proposition is based on the fact that not only glucose (Bazzigaluppi *et al.*, 2017; Vilela *et al.*, 2014), but also lactate (Proia *et al.*, 2016; Wyss *et al.*, 2011) are important fuels for the brain.

Because, it remains to be clarified whether lactate can be used to treat hypoglycemia, we evaluated the effects of the oral lactate on the occurrence of seizures and deaths during a period of five hours, among mice that received high doses of insulin.

Higher ($p < 0.05$) brain levels of lactate (Figure 2D: Control, Ins + Lac or Ins + Glc groups *versus* Ins group) were associated with the absence of seizures. In addition, the role of brain lactate to prevent seizures was reinforced by the fact that when seizures began, the brain levels of lactate in the Ins, Ins + Lac, or Ins + Glc groups were lower ($p < 0.05$) than those in the control group (Figure 2H). In agreement with these results, Wieggers *et al.* (2016) demonstrated that in type 1 diabetes patients, symptoms of

insulin-induced hypoglycemia are related to the decreased availability of lactate in the brain.

Interestingly, hypoglycemic mice that received oral glucose (Ins + Glc group) or lactate (Ins + Lac group) had similar brain lactate levels (Figure 2D), confirming that oral glucose is an important source of brain lactate (Bazzigaluppi *et al.*, 2017).

Despite the fact that glucose prolonged the time until the first seizure, mice that received glucose (Ins + glc group) or saline (Ins + saline group) had similar death rates (Tables I and II). Those results can be attributed to the fact that the dose of glucose was sufficient to prolong the time until the first seizure induced by the high doses (5 U/kg and 10.0 U/kg) of insulin but was not enough to prevent severe hypoglycemia.

The same dose of oral lactate that delayed the onset of first convulsion also increased hypoglycemia-induced mortality (Table II and Table III). One possible explanation for this finding is that lactate has a low K_m value, i.e., about 2.5 mM (Shulman, Hyder, Rothman, 2001), which is near its physiological level in blood. Therefore, there is a limitation in the transport of lactate through the blood-brain barrier and the elevated blood lactate levels cannot supply the brain lactate deficit due to hypoglycemia. As

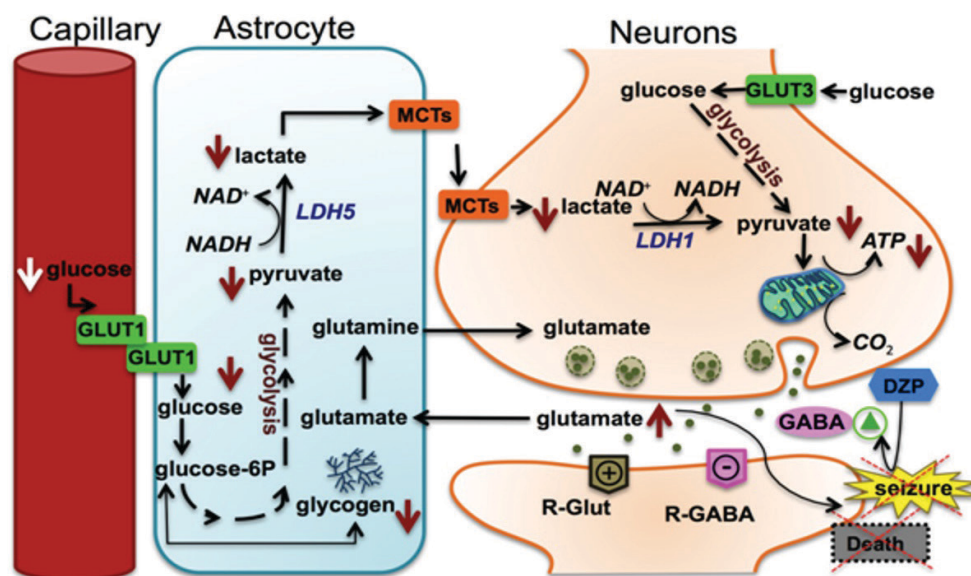


FIGURE 3 - Scheme based on the model proposed by Pellerin and Magistretti (2012). Our hypothesis is that during severe hypoglycemia, the availability of glucose to astrocytes becomes sharply decreased. Thus, the amount energy produced by glycolysis and the release of lactate to neurons also decreases. In contrast, the concentration of glutamate increases. Because diazepam decreases glutamate release by enhancing GABAergic inhibition of glutamatergic neurons, we suggest that an increased availability of excitatory neurotransmitters can trigger glutamate toxicity, and thereby promote seizures and deaths. Furthermore, the decreased availability of brain lactate cannot be compensated for lactate from blood, because the brain's ability for lactate uptake is very limited. Key: GABA, gamma-aminobutyric acid; R-GABA, GABA receptor; R-Glut, glutamate receptor; GLUT, glucose transporter; glucose 6-P, glucose 6 phosphate; MCTs, monocarboxylate transporters; DZP, diazepam; LDH, lactate dehydrogenase; +, stimulation; -, inhibition; ▲, potentiation.

consequence of this limitation, the elevation in blood lactate concentration after lactate administration (Figure 2C) was not followed by an elevation of brain lactate (Figure 2D). Moreover, the decrease in brain lactate levels which occurred during severe insulin induced hypoglycemia (Figure 2H) may promote neuronal death.

Taken together, the results of the present study, when combined with recent reports (Newman, Korol, Gold, 2011; Falkowska *et al.*, 2015; Boury-Jamot *et al.*, 2016) concerning the roles of glucose, lactate, and glutamate in brain metabolism, permit us formulate the hypothesis illustrated in Figure 3. Our hypothesis, based on the astrocyte-neuron lactate shuttle model (Pellerin, Magistretti, 2012, Shen *et al.*, 2014), states that during insulin-induced hypoglycemia, there is a sharp decrease in the availability of glucose to the astrocytes promoting decreased glycolysis in these cells and reduced release of lactate to the neurons. In other words, there is a disruption of the harmonic interaction between astrocytes (glycolysis, lactate release, and glutamate uptake) and neurons (lactate uptake and its use as a fuel source in oxidative metabolism). But, it must be emphasized that the interactions between astrocytes and neurons involve not only glucose/lactate metabolism, but also neuroprotective mechanisms similar than that triggered during ischemia (Gouix *et al.*, 2014), chronic hyperglycemia (Rivera-Aponte *et al.*, 2015), and traumatic brain injury (Crupi *et al.*, 2013).

Diazepam increases GABAergic inhibition of glutamatergic neurons (Lason, Chelebicka, Rejdak, 2013; Malhi *et al.*, 2014) promote an anticonvulsant effect (Table III). Therefore, we suggest that an elevated availability of excitatory neurotransmitters could have triggered seizures and deaths (Sulkowski, Dabrowska-Bouta, Struzynsk, 2013). Consistent with this hypothesis, it was previously proposed that excitatory amino acid pathways may mediate the insulin-induced hypoglycemic seizures in Swiss mice (Anuradha, Hota, Pandhi, 2004).

Finally, our results did not change the current view that lactate is the main energetic fuel to the neurons. However, in contrast with glucose, oral lactate intensifies insulin toxicity.

ACKNOWLEDGMENTS

This research was supported by PRONEX/CNPq/Fundação Araucária.

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Received for publication on 20th September 2017

Accepted for publication on 19th January 2018