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Short communication

Comparative effects of short-term and long-term insulin-induced hypoglycemia on glucose production in the perfused livers of weaned rats

Romir Rodrigues[1], Kassia P. S. Feitosa[2], Antonio M. Felisberto-Junior[3], Helenton C. Barrena[2], Rui Curi[4], Roberto B. Bazotte[2]

[1]Department of Pharmacy, UNIPAR, 87706-490, Paranavaí, PR, Brazil

[2]Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, PR, 87020-900, Brazil

[3]Department of Biology, UNINGA, Maringá, PR, Brazil

[4]Department of Physiology and Biophysics, University of São Paulo, 05508-900, São Paulo, SP, Brazil

Correspondence: Roberto B. Bazotte, e-mail: rbbazotte@uem.br

Abstract:
The liver glucose production (LGP) levels of 15-h overnight fasted weaned rats submitted to short-term insulin-induced hypoglycemia (ST-IIH) and long-term IIH (LT-IIH) were compared. Experiments to characterize ST-IIH or LT-IIH that followed an intraperitoneal (*ip*) injection (1.0 U/kg) of regular (ST-IIH) or insulin detemir (LT-IIH) were performed and glycemia were measured 0 (normoglycemic control), 0.5 h (ST-IIH), 4 h and 6 h (LT-IIH) later. The values of glycemia (mg/dl) were 77.8 ± 7.2 (normoglycemic control), 26.2 ± 6.1 (ST IIH 0.5 h), 21.2 ± 7.6 (LT-IIH 4 h) and 35.3 ± 14.5 (LT-IIH 6.0). The LGP levels were measured in the rats submitted to ST-IIH (0.5 h) and LT-IIH (4 h or 6 h). The rats that received *ip* saline were used as the normoglycemic control group (COG). The livers from the COG and IIH groups (ST-IIH or LT-IIH) were perfused *in situ* with infusion of L-alanine (5 mM), L-glutamine (10 mM), glutamine dipeptide (5 mM), L-lactate (2 mM) or glycerol (2 mM). The ST-IIH rats showed a higher LGP level than COG group following the L-glutamine infusion ($p < 0.05$), but the LGP levels that were measured following the L-lactate, L-alanine, glutamine dipeptide (5 mM), L-lactate (2 mM) or glycerol infusion remained unchanged. Moreover, if the period of IIH was expanded to 4 h following insulin injection, the LGP levels induced by L-alanine, glutamine dipeptide or glycerol infusion also increased ($p < 0.05$, LT-IIH *vs.* COG). However, the LGP from the L-lactate infusion remained unchanged until 6 h after insulin injection. In conclusion, these results suggest that the intensification of liver gluconeogenesis during ST-IIH and LT-IIH in weaned rats is not a synchronous "all or nothing" process; instead, this process integrated in a temporal manner and is specific for each gluconeogenic substrate.

Key words:
hypoglycemia, liver gluconeogenesis, weaned rats, insulin detemir

Abbreviations: AUC – area under the curve, COG – normoglycemic control group, IIH – experimental hypoglycemic group, *ip* – intraperitoneal, LGP – liver glucose production, LT-IIH – long-term insulin-induced hypoglycemia, NAD^+ – nicotinamide adenine dinucleotide (oxidized form), NADH – nicotinamide adenine dinucleotide (reduced form), ST-IIH – short-term insulin-induced hypoglycemia

Introduction

Insulin-induced hypoglycemia is common in the young children with type 1 diabetes, which may partially account for its high mortality rate in the 1–4

year-old age group [5]. For this reason, insulin-induced hypoglycemia is the rate-limiting step in achieving excellent control of glycemia in type 1 diabetic children [18].

Weaned rats submitted to insulin-induced hypoglycemia are a suitable experimental model of hypoglycemia in early age because, like diabetic children, they demonstrate a higher tendency to show severe insulin-induced hypoglycemia if compared with adult rats [19, 20].

Moreover, considering that liver gluconeogenesis is crucial to the maintenance of glycemia during insulin-induced hypoglycemia [6, 11, 13] and that the transition from the weaning to the adult age is an unfavorable condition for gluconeogenesis [4, 8], it is critical to investigate this metabolic pathway in weaned rats submitted to insulin-induced hypoglycemia.

The results of a previous study agree with this proposition [17]; the authors demonstrated that weaned rats submitted to short-term insulin-induced hypoglycemia showed a higher liver capacity for producing glucose from L-glutamine, but the liver gluconeogenesis of L-alanine, glutamine dipeptide, L-lactate and glycerol were unchanged. However, the metabolic changes that occurred during long term insulin-induced hypoglycemia were not investigated.

Therefore, to better elucidate the contribution of liver gluconeogenesis to the maintenance of glycemia during hypoglycemia in weaned rats, the activation of the liver glucose production levels during short-term and long-term insulin-induced hypoglycemia were compared.

Material and Methods

Materials

Insulin detemir (Levemir) was purchased from Novo Nordisk (São Paulo, SP, Brazil). L-lactate dehydrogenase was obtained from Sigma Chemical Company (St. Louis, MO, USA). L-glutamine, L-alanine, L-lactate, L-alanyl-L-glutamine (glutamine dipeptide) and all other reagents were received from the suppliers.

Animals

Male Wistar weaned rats (21–23 days) weighing approximately 50 g were used in the study. The manipulation followed the Brazilian law regarding the protection of animals and was performed with the permission of the state animal welfare committee. The rats were maintained under a constant temperature (23°C) with an automatically controlled photoperiod (12 h light/12 h dark). The rats had free access to water and food (standard commercial laboratory diet – Nuvilab®) until the day before the experiment when they were fasted overnight (15 h).

Short-term insulin-induced hypoglycemia (ST-IIH) and long-term IIH (LT-IIH)

ST-IIH or LT-IIH was induced with an intraperitoneal (*ip*) injection of regular (1 U/kg) or insulin detemir (1 U/kg). The dose was based on previous investigations [16, 17].

The glycemia was measured 0 at 0 h (normoglycemic control), 0.5 h (ST-IIH), 4 h (LT-IIH) and 6 h (LT-IIH) after the administration of the insulin detemir. The blood was collected by decapitation and immediately centrifuged to obtain the serum for the glucose determination.

Liver perfusion technique

Rats fasted overnight were anesthetized with an *ip* injection of sodium thiopental (40 mg/kg) at 0.5 h, 4 h or 6 h following the saline (normoglycemic control group, i.e., COG) or the insulin injection (experimental hypoglycemic rats, i.e., IIH group).

After a suitable level of anesthesia was achieved, the rats were submitted to a laparotomy. The livers were perfused *in situ* with a constant flux (4 ml/min × g) using Krebs Henseleit bicarbonate buffer, pH 7.4, that was saturated with O_2/CO_2 (95/5%). The perfusion fluid was pumped into a controlled temperature (37°C) membrane oxygenator prior to entering the liver *via* the portal vein. This experimental approach discarded the influence of hepatic glycogen catabolism, the changes in hepatic blood flux and the variability of blood glucose precursors [12].

The gluconeogenic substrates were L-alanine (5 mM), L-glutamine (10 mM), glutamine dipeptide (5 mM), L-lactate (2 mM) and glycerol (2 mM). The concentration of each gluconeogenic substrate was based on a previous study [17].

A typical liver perfusion experiment is illustrated in Figure 1. Following a pre-infusion period (10 min), the gluconeogenic substrate was dissolved in the perfusion fluid and was infused for 40 min; this was followed by a period of post-infusion (10 min). The sam-

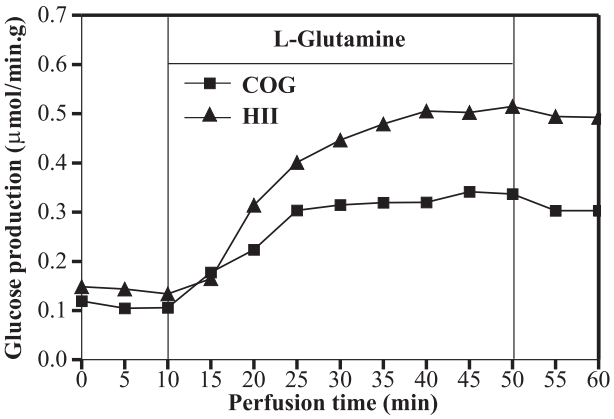

Fig. 1. Demonstrative experiments of glucose production from glutamine (10 mM) in the perfused livers of 15-h fasted weaned rats that received insulin (IIH) or saline (COG). The effluent perfusate was sampled at 5-min intervals and was used for glucose determination

ples of the effluent perfusion fluid were collected at 5-min intervals and the concentration of the glucose was determined using a lab kit test (Labtes®).

The kinetics of the glucose production from a gluconeogenic substrate are illustrated in Figure 1. After the infusion of the glucose precursor was initiated, the glucose production increased progressively. The differences in the glucose production during and before the infusion of the gluconeogenic substrate allowed for the calculation of the area under the curves (AUCs), which are expressed as mol/g. The AUCs that are shown in Tables 1–3 were obtained from similar experiments as the experiment that is illustrated in Figure 1.

In a portion of the experiments, the liver production of pyruvate and L-lactate were measured from the effluent perfusion fluid. The concentrations of pyruvate [3] and L-lactate [9] were measured with standard enzymatic techniques that utilized lactate dehydrogenase.

The liver perfusion experiments were performed on pairs of rats; one rat was from the IIH group and other served as a COG. Therefore, the ability of the liver to produce glucose from L-glutamine, L-alanine, glutamine dipeptide, L-lactate and glycerol from the rats that were submitted to IIH (ST-IIH or LT-IIH) or COG were compared.

Statistical analysis

The data were analyzed with an unpaired Student's *t*-test or by ANOVA using the Graph-Pad Prism software (version 5.0). The results are reported as the means ± the standard deviation of the means (SD); $p < 0.05$ was considered to be statisticallysignificant.

Result

The values (means ± SD, n = 5) that were obtained for glycemia at 0 h (Control normoglycemic), 0.5 h (ST-IIH), 4 h (LT-IIH) and 6 h (LT-IIH) after insulin administration were 77.8 ± 7.2, 26.2 ± 6.1, 21.2 ± 7.6 and 35.3 ± 14.5 mg/dl, respectively.

The results from the *in situ* liver perfusion experiments are presented in Tables 1–3.

Table 1 shows that the production of glucose, pyruvate and L-lactate from L-alanine was unchanged at 0.5 h (ST-IIH) after the insulin administration. However, the livers from the IIH group showed higher

Tab. 1. Glucose, pyruvate and L-lactate production from L-alanine (5 mM) in the livers of the normoglycemic control group (COG) and the hypoglycemic (IIH) group of 15-h fasted rats. The liver experiments were performed at 0.5 h and 4.0 h after the administration of saline (COG) or insulin (IIH group). The areas under the curves (AUCs) were obtained as described in the Materials and Methods and in Figure 1. The AUCs values (μmol/g) were expressed as the means ± SD of 4–7 liver perfusion experiments

	AUC (μmol/g) from L-alanine					
	Glucose production		Pyruvate production		L-lactate production	
	COG	IIH	COG	IIH	COG	IIH
0.5-h	4.2 ± 2.2	5.7 ± 2.0	2.2 ± 1.0	2.3 ± 1.2	6.8 ± 1.6	7.5 ± 2.2
4.0-h	3.9 ± 1.1	6.9 ± 1.3*	8.7 ± 1.3	13.9 ± 1.5*	10.0 ± 2.1	14.6 ± 1.7*

* $p < 0.05$ *vs.* COG

Tab. 2. Glucose and pyruvate production from L-lactate (2 mM) in the livers of the normoglycemic control group (COG) and the hypoglycemic (IIH) group of 15-h fasted rats. The liver experiments were performed at 0.5 h, 4.0 h and 6.0 h after the administration of saline (COG) or insulin (IIH group). The areas under the curves (AUCs) were obtained as described in the Materials and Methods and in Figure 1. The AUCs values (μmol/g) were expressed as the means ± SD of 4–6 liver perfusion experiments

AUC (μmol/g) from L-lactate (μmol/g)		
	COG	IIH
Glucose production 0.5-h	12.7 ± 6.2	12.0 ± 3.7
Pyruvate production 0.5-h	2.9 ± 1.6	2.8 ± 1.8
Glucose production 4.0-h	19.1 ± 5.0	20.6 ± 3.4
Pyruvate production 4.0-h	7.2 ± 0.9	8.5 ± 1.1
Glucose production 6.0-h	12.0 ± 1.8	15.4 ± 4.1
Pyruvate production 6.0-h	9.2 ± 1.5	8.8 ± 3.6

$p > 0.05$ *vs.* COG for all comparisons

Tab. 3. Glucose production from L-glutamine (10 mM), glutamine dipeptide (5 mM) and glycerol (2 mM) in the livers of the normoglycemic control group (COG) and the hypoglycemic (IIH) group of 15-h fasted rats. The liver experiments were performed at 0.5 h or 4.0 h after the administration of saline (COG) or insulin (IIH group). The areas under the curves (AUCs) were obtained as described in the Materials and Methods and in Figure 1. The AUCs values (μmol/g) were expressed as the means ± SD of 3–6 liver perfusion experiments

AUC (μmol/g) from L-glutamine, glutamine dipeptide and glycerol		
	COG	IIH
L-Glutamine		
0.5-h	3.8 ± 1.2	6.9 ± 1.8*
4.0-h	3.2 ± 1.4	5.0 ± 1.5*
Glutamine dipeptide		
0.5-h	1.6 ± 0.5	1.5 ± 0.6
4.0-h	0.8 ± 0.2	2.6 ± 1.2*
Glycerol		
0.5-h	10.6 ± 3.4	11.7 ± 2.8
4.0-h	9.6 ± 1.4	12.9 ± 2.3*

* $p < 0.05$ *vs.* COG

($p < 0.05$) glucose, pyruvate and L-lactate production from L-alanine at 4.0 h (LT-IIH) after the insulin administration (IIH *vs*. COG).

As shown in Table 2, neither the glucose nor the pyruvate production from L-lactate was changed at 0.5 h (ST-IIH) or 4 h (LT-IIH) after the insulin administration (IIH *vs.* COG) and the values remained unchanged when the period of IIH was expanded to 6 h (LT-IIH) after the insulin administration.

The livers from the IIH rats showed higher ($p < 0.05$) levels of glucose production from L-glutamine at 0.5 h (ST-IIH) and 4 h (LT-IIH) after insulin administration (IIH *vs.* COG). However, higher ($p < 0.05$ *vs.* COG) levels of glucose production from the glutamine dipeptide or glycerol was measured at 4 h (LT-IIH), but not at 0.5 h (ST-IIH), after the insulin was administered (Tab. 3).

Discussion

Because the IIH was well established at 0.5 h (ST-IIH) and maintained until 6 h (LT-IIH) after insulin administration, the liver gluconeogenesis in rats submitted to ST-IIH and LT-IIH was investigated.

Because L-alanine utilizes all of the steps of the gluconeogenic pathway [14], this amino acid was the first liver glucose precursor that was investigated. In addition, because gluconeogenesis depends on the oxygen supply and on several cellular compartments (the plasma membrane, cytosol, mitochondria and microsomal fraction), the glucose, L-lactate and pyruvate that are released as byproducts of L-alanine catabolism can be used as markers of the integrity of the hepatocyte [16].

L-alanine infused in the liver crosses the cell membrane and is then converted to pyruvate. From the cytosol, pyruvate enters mitochondria where it is carboxylated and then leaves the mitochondria as malate [7]. In the cytosol, malate is converted to oxaloacetate and then to phosphoenolpyruvate and after various steps, is converted by microsomal glucose-6-phosphatase to glucose, which is released from the hepatocyte [1]. As expected, an increased glucose production was observed following the infusion of L-alanine. In contrast with the adult rats [13], the production of glucose, pyruvate and L-lactate from L-alanine in the livers of the weaned rats submitted to ST-IIH (0.5 h) were not different if compared with COG. However, the glucose production from L-alanine increased at 4 h (LT-IIH) after insulin administration (IIH *vs.* COG).

This result may be partly attributed to the increased catabolism of L-alanine because the levels of L-lactate and pyruvate released from this amino acid were increased (Tab. 1).

In the second set of experiments, which used L-lactate as a gluconeogenic substrate, we observed that the production of glucose and pyruvate production were unchanged at both 0.5 h (ST-IIH) and 4 h (LT-IIH) following insulin administration (IIH *vs.* COG). For this reason, the period of the IIH was expanded to last until 6 h following the insulin injection. Nonetheless, the LGP from L-lactate did not change (Tab. 2).

In the third set of experiments, the liver gluconeogenesis from L-glutamine, which enters the gluconeogenesis pathway after the pyruvate carboxylase step and before the phosphoenolpyruvate carboxykinase step [17], was investigated. The results showed an increased ($p < 0.05$ IIH *vs.* COG) glucose production during the ST-IIH (0.5 h after insulin administration), which was maintained during the LT-IIH (4 h after the insulin administration).

In the next set of experiments, the liver gluconeogenesis from glutamine dipeptide or glycerol were investigated. The results were similar to those observed for L-alanine; the glucose production at 0.5 h (ST-IIH) after insulin injection was unchanged (IIH *vs.* COG) and the glucose production at 4 h (LT-IIH) after insulin administration increased ($p < 0.05$ IIH *vs.* COG) (Tab. 3).

The mechanism of the higher glucose production ability during IIH likely involves an increased release of counter-regulatory hormones, which can activate the enzymes alanine aminotransferase, glutaminase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [2, 15], which overcome the inhibitory effect of the insulin on hepatic gluconeogenesis.

From the liver perfusion experiments that were described above (Tabs. 1–3), it is possible to establish a time sequence of the changes that occur during gluconeogenesis for different glucose precursors during ST-IIH and LT-IIH. The process begins with the L-lactate that enters the gluconeogenesis pathway as pyruvate [7] and uses all the steps of gluconeogenesis. The results suggest that the gluconeogenesis that occurred during ST-IIH and LT-IIH was not activated or inhibited, but rather, it was maintained. Furthermore, because the conversion of L-lactate to pyruvate depends on the activity of lactate dehydrogenase and the potential of cytosolic NADH: NAD^+ [10], we can infer that these variables remained unchanged in the IIH weaned rats.

It is noteworthy that L-glutamine was the unique liver gluconeogenic substrate that showed an increased ability to produce glucose during ST-IIH (0.5 h after insulin injection). This result raises the following question: why did the livers from the IIH rats show increased glucose production from L-glutamine at 0.5 h following the administration of insulin but not from L-alanine or glycerol?

Before answering this question, it is necessary to consider that L-alanine, which enters the gluconeogenesis pathway as pyruvate, utilizes all of the steps of this metabolic pathway. Therefore, the superior metabolic performance of L-glutamine could be related to its entrance in the gluconeogenic pathway following pyruvate carboxylase. In other words, L-glutamine may bypass the pyruvate carboxylase step. However, glycerol, which bypasses both the pyruvate carboxylase and the phosphoenolpyruvate carboxykinase steps, did not show an increased liver glucose production at 0.5 h after insulin administration. Another interesting possibility is that the mitochondrial glutaminase [15] is more intensely activated in the livers of hypoglycemic weaned rats. In agreement with this proposition and as shown by the demonstrative experiment that is depicted in Figure 1, steady-state conditions of glucose production were attained approximately 15 and 30 min after the infusion of L-glutamine in the COG and IIH groups was initiated, respectively. Moreover, because the higher gluconeogenesis from glycerol occurred at 4 h, but not at 0.5 h, after insulin injection, the possibility of the activation of fructose-1,6-bisphosphatase, which is the main enzyme between the phosphoenolpyruvate carboxykinase and glucose- 6-phosphatase steps, must be considered.

In conclusion, these results suggest that the intensification of liver gluconeogenesis during ST-IIH and LT-IIH in weaned rats was not a synchronous "all or nothing" process; instead, this process was integrated in a temporal manner and was specific for each gluconeogenic substrate.

Finally, our results have great clinical interest because they help to elucidate the complexity of the condition of ST-IIH and LT-IIH in weaned rats and provide additional information regarding the predisposition to severe hypoglycemia in the young children.

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References:

1. Bassoli BK, Cassolla P, Murad GRB, Constantin J, Salgueiro-Pagadigorria CL, Bazotte RB, Silva RS, Souza HM: Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: effects on hepatic glucose release and glycaemia. Cell Biochem Func, 2008, 26, 320–328.
2. Cynober LA: Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. Nutrition, 2002, 18, 761–766.
3. Czok R, Lamprecht W: Pyruvate, phosphoenolpyruvate and D-glycerate-2-phosphate. In: Methods of Enzymatic Analysis. Ed. Bergmeyer HU, Academic Press, New York, 1974, 1446–1448.
4. Decaux JF, Ferré P, Girard J: Effect of weaning on different diets on hepatic gluconeogenesis in the rat. Biol Neonate, 1986, 50, 331–336.
5. Dickerman MJ, Jacobs BR, Vinodrao H, Stockwell DC: Recognizing hypoglycemia in children through automated adverse-event detection. Pediatrics, 2011, 127, 1035–1041.
6. Felisberto-Junior AM, Manso F, Gazola VAFG, Obici S, Geisler SA, Bazotte RB: Oral glutamine dipeptide prevents against prolonged hypoglycaemia induced by Detemir insulin in rats. Biol Pharm Bull, 2009, 32, 232–236.
7. Geisler SA, Felisberto-Junior AM, Tavoni TM, Carrara MA, Curi R, Bazotte RB. Participation of the liver gluconeogenesis in the glibenclamide induced hypoglycaemia in rats. Cell Biochem Funct, 2011, 29, 81 –86.
8. Girard J, Perdereau D, Narkewicz M, Coupé C, Ferré P, Decaux JF, Bossard P: Hormonal regulation of liver phosphoenolpyruvate carboxykinase and glucokinase gene expression at weaning in the rat. Biochimie, 1991, 73, 71–76.
9. Gutmann I, Wahlefeld W: L-(+)-lactate. Determination with lactate dehydrogenase and NAD. In: Methods of Enzymatic Analysis, Ed. Bergmeyer HU, Academic Press, New York, 1974, 1464–1472.
10. Hartmann EM, Garcia RF, Gazola VAFG, Barrena HC, Bazotte RB: Investigation of glycemia recovery with oral administration of glycerol, pyruvate, and L-lactate during long-term, insulin-induced hypoglycemia. J Diabetes Complications, 2010, 24, 301–305.
11. Malta A, Furlan MP, Vitoriano AS, Barrena HC, Bazotte RB, Gazola VG. Insulin sensitivity and liver glucose production in the rat are influenced by lifetime food restriction. Nutr Res, 2010, 30, 626–31.
12. Mario EG, Leonardo ES, Bassoli BK, Cassolla P, Borba-Murad GRB, Bazotte RB, Souza HM: Investigation of the acute effect of leptin on the inhibition of glycogen catabolism by insulin in rat liver perfused in situ. Pharmacol Rep, 2009, 319–324.
13. Nascimento KF, Garcia RF, Gazola VAFG, Souza HM, Obici S, Bazotte RB. Contribution of hepatic glycogenolysis and gluconeogenesis in the defense against short-term insulin induced hypoglycemia in rats. Life Sci, 2008, 82, 1018–1022.
14. Newsholme P, Abdulkader F, Rebelato E, Romanatto T, Pinheiro CH, Vitzel KF, Silva EP et al.: Amino acids and diabetes: implications for endocrine, metabolic and immune function. Front Biosci, 2011, 6:315–39.
15. Nissim I, Horyn O, Luhovyy B, Lazarow A, Daikhin Y, Nissim I, Yodkoff M: Role of the glutamate dehydrogenase reaction in furnishing aspartate nitrogen for urea synthesis: studies in perfused rat liver with ^{15}N. Biochem J, 2003, 376, 179–188.
16. Obici S, Lopes-Bertolini G, Curi R, Bazotte RB: Liver glycogen metabolism during short-term insulin-induced hypoglycemia in fed rats. Cell Biochem Funct, 2008, 26, 755–759.
17. Oliveira-Yamashita F, Garcia RF, Felisberto-Junior AM, Curi, R, Bazotte RB: Evidence that L-glutamine is better than L-alanine as gluconeogenic substrate in perfused liver of weaned fasted rats submitted to short-term insulin-induced hypoglycaemia. Cell Biochem Funct, 2009, 27, 30–34.
18. Shalitin S, Phillip M: Hypoglycaemia in type 1 diabetes: a still unresolved problem in the era of insulin analogs and pump therapy. Diabetes Care, 2008, 31, S121–S124.
19. Yamada KA, Rensing N, Izumi Y, De Erausquin GA, Gazit V, Dorsey DA, Herrera DG. Repetitive hypoglycemia in young rats impairs hippocampal long-term potentiation. Pediatr Res, 2004, 55, 372–9.
20. Yamada KA, Rensing N, Thio LL RB: Ketogenic diet reduces hypoglycemia-induced neuronal death in young rats. Neurosci Lett, 2005, 385, 210–214.

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