Original Research

Paradoxical increase in liver ketogenesis during long-term insulin-induced hypoglycemia in diabetic rats

Fabiana P M Schiavon[1], Vilma A F G Gazola[1], Maria M D P Furlan[1], Helenton C Barrena[2] and Roberto B Bazotte[2]

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

Corresponding author: Dr Roberto B Bazotte. Email: rbbazotte@uem.br

Abstract

It is well established that insulin inhibits liver ketogenesis. However, during insulin-induced hypoglycemia (IIH) the release of counterregulatory hormones could overcome the insulin effect on ketogenesis. To clarify this question the ketogenic activity in livers from alloxan-diabetic rats submitted to long-term IIH was investigated. Moreover, liver glycogenolysis, gluconeogensis, ureagenesis and the production of L-lactate were measured, and its correlation with blood levels of ketone bodies (KB), L-lactate, glucose, urea and ammonia was investigated. For this purpose, overnight fasted alloxan-diabetic rats (DBT group) were compared with control non-diabetic rats (NDBT group). Long-term IIH was obtained with an intraperitoneal injection of Detemir insulin (1 U/kg), and KB, glucose, L-lactate, ammonia and urea were evaluated at 0, 2, 4, 6, 8 or 10 h after insulin injection. Because IIH was well established two hours after insulin injection this time was used for liver perfusion experiments. The administration of Detemir insulin decreased ($P < 0.05$) blood KB and glucose levels, but there was an increase in the blood L-lactate levels and a rebound increase in blood KB during the glucose recovery phase of IIH. In agreement with these results, the capacity to produce KB from octanoate was increased in the livers of DBT rats. Moreover, the elevated blood L-lactate levels in DBT rats could be attributed to the higher ($P < 0.05$) glycogenolysis when part of glucose from glycogenolysis enters glycolysis, producing L-lactate. In contrast, except glycerol, gluconeogenesis was negligible in the livers of DBT rats. Therefore, during long-term IIH the higher liver ketogenic capacity of DBT rats increased the risk of hyperketonemia. In addition, in spite of the fact that the insulin injection decreased blood KB, there was a risk of worsening lactic acidosis.

Keywords: ketogenesis, hypoglycemia, experimental diabetes, Detemir insulin

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Introduction

It is well established that in certain physiological conditions including fasting, pregnancy, hyperlipidic diet and prolonged exercise, a modest ketonemia elevation seems to be important as an energy source to all extrahepatic tissues including the brain.[1] Conversely, diabetic ketoacidosis is a potentially life-threatening complication that happens predominantly in type 1 diabetes.[2–5] Nevertheless, despite major advances obtained among clinicians and biochemists, a number of fundamental issues concerning the hormonal and biochemical mechanisms involved in the liver control of ketogenesis during diabetic ketoacidosis, particularly the concomitant blood increase of lactic acid[6] remain to be clarified.

Moreover, studies of ketogenesis during hypoglycemia in the diabetic condition are scarce.[7] This question acquires greater relevance when considering that hypoglycemia induced by excess of administered insulin and hyperketonemia developed as a consequence of severe insulin deficiency are the main acute complication of type 1 diabetes. Furthermore, type 1 diabetic patients could show a clinic hyperketonemia posthypoglycemia.[7,8]

In addition, it is well established that insulin inhibits liver ketogenesis. However, during hypoglycemia the release of counterregulatory hormones[9] could overcome the insulin effect on ketogenesis. To clarify this question and considering that alloxan-diabetic rats are a suitable rat model of type 1 diabetes,[10,11] our laboratory investigated the hepatic ketogenesis in diabetic rats submitted to short-term insulin-induced hypoglycemia (IIH).[12,13] The results demonstrated that the ketogenic capacity was maintained in the livers of diabetic rats.[13] Nonetheless, considering

the slow activation of the key enzymes of ketogenesis, the possibility of an increased activity of this metabolic pathway during long-term IIH must be considered.

Therefore, in this study, we shall focus on the liver ketogenesis during long-term hypoglycemia in diabetic rats, i.e. a biochemistry study with clinical implications. Moreover, liver production of glucose (from glycogenolysis and gluconeogenesis), L-lactate (from glycolysis), urea (from catabolism of amino acids) and their correlation with blood levels of ketone bodies (KB), L-lactate, glucose, urea and ammonia were investigated.

Material and methods

Materials

Detemir insulin (Levemir®) was purchased from Novo Nordisk (Sao Paulo, SP, Brazil). β-Hydroxybutyrate dehydrogenase and alloxan were obtained from Sigma Chemical Company (St Louis, MO, USA). Octanoate was purchased from Acros Organics (Morris Plains, NJ, USA). Glycerol, L-lactate, L-alanine pyruvate and all other reagents were of the highest purity obtainable.

Animals

Male Wistar rats (*Rattus norvegiccus*), weighing 180–220 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). Water and a standard commercial laboratory diet (Nuvilab®, Nuvital Nutrientes S/A, Colombo, PR, Brazil) were given *ad libitum* before the experimental procedures. Diabetes (DBT group) was induced by an intravenous injection of alloxan (40 mg/kg dissolved in saline), and four days later blood samples were then taken from a cut on the tip of the tail to determine the glycemia.[14] Control non-diabetic rats (NDBT group) received an equal volume of saline and the same treatment as that of the DBT group. All animals of the DBT group with glycemia above 16 mmol/L during the fed state were included in the study. On the day before the experiment, the DBT and NDBT groups were food-deprived from 17:00 hours. All experiments were performed after an overnight fasting (17:00–08:00 hours). The manipulation followed the Brazilian law on the protection of animals and was performed with permission of the state animal welfare committee.

Long-term IIH

Long-term IIH in DBT and NDBT rats was obtained with Detemir insulin (not diluted). Detemir insulin (1.0 U/kg) was intraperitoneally injected with the help of an infusion pump (Insight®, Insight Pesquisa e Ensino, Ribeirao Preto, SP, Brazil).

In vivo experiments

The rats were anesthetized with intraperitoneal thiopental (40 mg/kg) 0, 2, 4, 6, 8 or 10 h after insulin injection. After laparotomy, blood was collected from the cava vein for glucose,[14] L-lactate,[15] ammonia,[16] urea[16] and KB (acetoacetate[17] plus β-hydroxybutyrate[18]) measurements.

Liver perfusion experiments

IIH was well established two hours after Detemir insulin administration. Therefore, at this time the rats were anesthetized with intraperitoneal sodium thiopental (40 mg/kg). After laparotomy, blood was collected from the cava vein for glucose determination,[14] which is necessary to confirm IIH. Immediately, a cannula was inserted into the portal vein for *in situ* liver perfusion. The perfusion fluid was Krebs-Henseleit (KH) bicarbonate buffer (pH 7.4) saturated with a mixture of O_2/CO_2 (95%/5%). Perfusion fluid was pumped through a temperature-controlled (37°C) membrane oxygenator before entering into the liver through the portal vein. In addition, a second cannula was inserted into the infrahepatic portion of the cava vein to collect the effluent perfusion fluid. The perfusion was done in an open system, without recirculation of perfusate (single pass). At the end the liver was removed and weighed, to allow precise metabolic calculations and the correction of flow rates.

Figure 1 shows a demonstrative experiment in which after a preinfusion period (10 min), glycerol was dissolved in the KH and infused during 20 min, followed by a postinfusion period (10 min without glycerol) to allow the return of basal values of the preperfusion period. Samples of the effluent perfusion fluid were collected from the inferior cava vein each 5 min and the glucose concentration was measured. Glucose production during the preinfusion period represents the glucose from endogenous glycogen (glycogenolysis) and the difference between the glucose production during and before glycerol infusion represents the glucose production from gluconeogenesis. The areas under the curves (AUC) of the preinfusion period

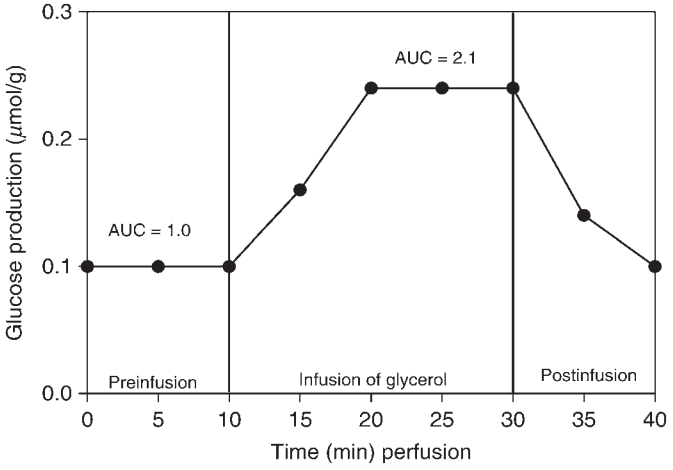

Figure 1 Demonstrative experiment of glucose prodution in *in situ* perfused liver. The effluent perfusate was sampled at five-minute intervals and used for the determination of glucose. Glucose production during the preinfusion period represents the glucose from endogenous glycogen (glycogenolysis) and the difference between the glucose production during and before glycerol (0.5 mmol/L) infusion represents the glucose production from gluconeogenesis. The areas under the curves (AUC) of the preinfusion period and infusion period were expressed as μmol/g

Table 1 Glucose production (μmol/g)

	L-alanine		L-lactate		Pyruvate		Glycerol	
	NDBT	DBT	NDBT	DBT	NDBT	DBT	NDBT	DBT
0.25 mmol/L	Nd	Nd	Nd	Nd	Nd	Nd	Nd	$0.45 \pm 0.15^*$
0.5 mmol/L	Nd	Nd	Nd	Nd	$3.61 \pm 0.70^*$	ND	$3.25 \pm 0.18^*$	2.65 ± 0.38
1.0 mmol/L	$1.25 \pm 0.05^*$	ND	$4.87 \pm 0.23^*$	ND	5.14 ± 0.97	ND	3.90 ± 0.28	$0.67 \pm 0.12^*$
2.0 mmol/L	2.71 ± 0.45	ND	6.86 ± 0.73	ND	$3.88 \pm 0.76^*$	ND	$3.30 \pm 0.02^*$	$0.38 \pm 0.01^*$
3.0 mmol/L	$1.64 \pm 0.25^*$	ND	6.06 ± 1.26	ND	Nd	Nd	Nd	Nd
5.0 mmol/L	$1.46 \pm 0.26^*$	ND	$4.95 \pm 0.75^*$	ND	Nd	Nd	Nd	Nd

ND: not detected; Nd: not determined

Liver production of glucose from increasing concentrations of L-alanine, L-lactate, pyruvate and glycerol in perfused livers of non-diabetic (NDBT) and diabetic (DBT) rats 2 h after intraperitoneal administration of Detemir insulin (1 U/kg) in 15 h fasted rats. The area under curve values (μmol/g) obtained as described in Materials and Methods were expressed as means $\pm$ SD of six-to-eight liver perfusion experiments

$^*P < 0.05$ when compared with the highest value in the same column

(glycogenolysis) and infusion period (gluconeogenesis) were expressed as μmol/g (Figure 1).

In addition to glucose, in part of the experiments the liver production of L-lactate,[15] urea[16] and pyruvate[19] were also evaluated.

Moreover, the maximal hepatic capacity to produce glucose from L-alanine, pyruvate, L-lactate or glycerol was obtained with increasing concentrations of L-alanine (1.0, 2.0 and 3.0 mmol/L), pyruvate (0.5, 1.0 and 2.0 mmol/L), L-lactate (1.0, 2.0, 3.0 and 5.0 mmol/L) or glycerol (0.25, 0.5, 1.0 and 2.0 mmol/L). The addition of these substances increased the rate of glucose production until the saturating concentration (i.e. the lowest concentration that produced the maximal hepatic glucose production) had been reached. The saturating concentrations of L-alanine, L-lactate, pyruvate and glycerol to the NDBT group were 2, 2, 1 and 1 mmol/L, respectively. Conversely, in the DBT group, the glucose production from L-alanine, L-lactate and pyruvate was negligible. With respect to glycerol, the maximal hepatic glucose production was obtained with a concentration of 0.5 mmol/L. These values (Table 1) were used in the liver perfusion experiments.

The basal rates of ketogenesis and the rates of ketogenesis during the infusion of octanoate (0.3 mmol/L) were expressed as AUC and the resulting data expressed as μmol/g as shown by the demonstrative experiment depicted in Figure 2. Moreover, the maximal liver capacity to produce KB from octanoate was obtained in a previous study[13] using the same procedure previously described for gluconeogenesis.

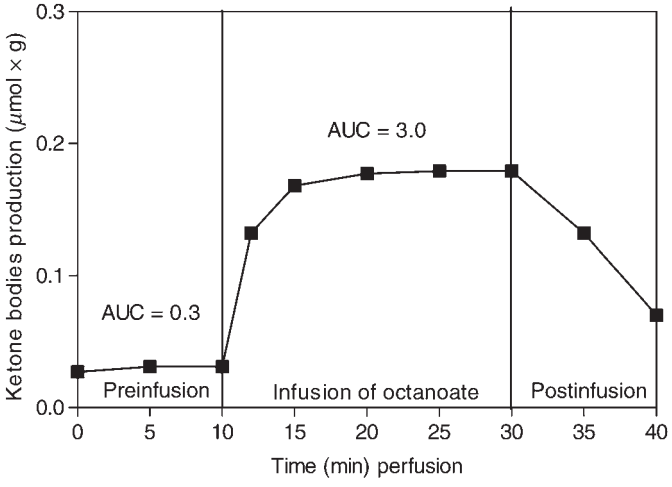

Figure 2 Demonstrative experiment of ketone bodies prodution in *in situ* perfused liver. The effluent perfusate was sampled at five-minute intervals and used for determination of ketone bodies (acetoaetate plus β-hydroxybutyrate). Ketone bodies production during the preinfusion period represents the basal ketogenesis from endogenous fatty acids and the difference between the ketone bodies production during and before octanoate (0.3 mmol/L) infusion represents the ability of the liver to produce ketone bodies from ketogenic precursors. The areas under the curves (AUC) of the preinfusion period and infusion period were expressed as μmol/g

Statistical analysis

Statistical analyses were performed with unpaired Student's *t*-test or analysis of variance, using the Graph-Pad Prism softwere (version 5.0). The level of significance was set at $P < 0.05$. The results were reported as means $\pm$ standard deviation (SD) of six to eight individual experiments.

Results

The results of blood levels of glucose, L-lactate, urea, ammonia and KB were summarized in Table 2.

The DBT group showed higher ($P < 0.05$ versus NDBT) glycemia than the NDBT group before insulin injection (time 0 h). Moreover, IIH was well established two hours ($P < 0.05$ versus time 0 h) after insulin administration in both the groups. Furthermore, glycemia recovery was observed eight hours after insulin administration to the NDBT group. In contrast, the DBT group showed partial glycemia recovery (Table 2).

Blood L-lactate concentration was higher in the DBT group ($P < 0.05$ versus NDBT) than in the NDBT group before insulin injection (time 0 h). After insulin injection, the blood levels of L-lactate increased ($P < 0.05$) in both groups. These changes were more intense ($P < 0.05$) in the DBT group (DBT versus NDBT groups). However, L-lactate concentration returned to basal values six hours (NDBT group) and eight hours (DBT group) after insulin injection (Table 2).

Blood urea concentration was higher in the DBT group ($P < 0.05$ versus NDBT) before (time 0 h) and after insulin injection (2, 4, 6, 8, 10 h). After insulin injection, blood urea levels were maintained in the DBT group (Table 2). Nevertheless, in the NDBT group (Table 2) urea levels

Table 2 Blood levels (mmol/L)

	0 h	2 h	4 h	6 h	8 h	10 h
Glucose NDBT	4.9 ± 0.27	1.2 ± 039*	1.3 ± 0.31*	2.8 ± 0.73*	4.4 ± 0.57	4.7 ± 0.82
Glucose DBT	7.2 ± 0.88†	2.4 ± 0.40*†	2.0 ± 0.53*†	2.6 ± 0.60*	5.0 ± 0.82*	5.4 ± 0.60*
L-lactate NDBT	1.4 ± 0.03	1.6 ± 0.36*	1.5 ± 0.02*	1.3 ± 0.07	1.5 ± 0.09	1.4 ± 0.10
L-lactate DBT	1.5 ± 0.08†	2.1 ± 0.24*†	3.2 ± 0.64*†	2.1 ± 0.22*†	1.6 ± 0.17	1.5 ± 0.21
Urea NDBT	6.3 ± 0.42	4.2 ± 0.49*	4.3 ± 0.81*	4.4 ± 0.46*	7.1 ± 0.63*	7.8 ± 0.82*
Urea DBT	10.2 ± 0.20†	10.3 ± 0.25†	10.2 ± 1.06†	10.6 ± 0.41†	10.5 ± 0.46†	10.1 ± 0.71†
Ammonia NDBT	0.62 ± 0.05	0.50 ± 0.03*	0.46 ± 0.03*	0.40 ± 0.02*	0.51 ± 0.04*	0.50 ± 0.03*
Ammonia DBT	0.75 ± 0.04†	0.65 ± 0.05*†	0.63 ± 0.02*†	0.69 ± 0.03†	0.72 ± 0.04†	0.69 ± 0.03†
KB NDBT	0.10 ± 0.00	0.07 ± 0.00*	0.08 ± 0.01*	0.11 ± 0.01	0.12 ± 0.02*	0.14 ± 0.03*
KB DBT	0.16 ± 0.02†	0.10 ± 0.01*†	0.10 ± 0.01*†	0.13 ± 0.02*	0.14 ± 0.02	0.21 ± 0.01*†

Blood levels of glucose, L-lactate, urea, ammonia and ketone bodies (KB), i.e. β-hydroxybutyrate plus acetoacetate, 0, 2, 4, 6, 8 and 10 h after intraperitoneal administration of Detemir insulin (1 U/kg) in 15 h fasted non-diabetic (NDBT) rats and diabetic (DBT) rats. The results were expressed as means ± SD of six to eight experiments

*$P < 0.05$ when compared with time 0 h. †$P < 0.05$ when compared with DBT versus NDBT (each parameter at a specific time after insulin administration)

decreased until four hours after insulin injection and increased ($P < 0.05$) again (10 versus 0 h after insulin administration).

The DBT group showed higher ($P < 0.05$ versus NDBT) blood ammonia concentration before (time 0 h) and after insulin injection (2, 4, 6, 8, 10 h). Moreover, after insulin administration, the blood ammonia concentration decreased ($P < 0.05$) in both groups (Table 2).

Blood KB levels were higher in the DBT group ($P < 0.05$ versus NDBT) before insulin injection (time 0 h). After insulin injection, blood KB levels decreased ($P < 0.05$) in both groups. However, from eight hours (NDBT group) and 10 h (DBT group) after insulin injection the blood KB reached higher values ($P < 0.05$) than before insulin injection (0 h).

Liver perfusion experiments two hours after insulin administration in the livers of NDBT and DBT rats were performed and the results were summarized in Tables 3–5.

Livers from the DBT group showed higher ($P < 0.05$) basal glucose (glycogenolysis), L-lactate and urea production. However, the basal liver KB production remained unchanged (Table 3).

As expected, the liver ketogenic activity increased ($P < 0.05$) during the infusion of octanoate. In addition, the differences before and during the infusion of octanoate result in the AUC depicted in Table 4. The results showed that the rates of ketogenesis (acetoatate plus β-hydroxybutyrate) were higher in the liver of diabetic hypoglycemic rats (DBD versus NDBD group).

Livers of the DBT group showed lower ($P < 0.05$ versus NDBT) glucose production from glycerol and negligible glucose production from the saturating concentrations of L-alanine, L-lactate and pyruvate (Table 5). In contrast to the results for glucose production, the DBT group showed higher ($P < 0.05$ versus NDBT) urea, L-lactate and pyruvate production from the saturating concentration of L-alanine.

Finally, livers from DBT rats infused with the saturating concentration of L-lactate showed higher ($P < 0.05$ versus NDBT) pyruvate production. In contrast, livers from DBT rats infused with the saturating concentration of pyruvate showed lower ($P < 0.05$ versus NDBT) L-lactate production.

Discussion

Long-term IIH can be achieved with the administration of a pharmacological dose of Detemir insulin.[20] Therefore, as we expected, hypoglycemia with at least six hours of duration was obtained (Table 2). Additionally, considering that IIH was well established two hours after insulin injection, this time was used to evaluate not only the liver ketogenesis but also the liver production of glucose (from glycogenolysis and gluconeogenesis), L-lactate (from glycolysis), urea (from catabolism of amino acids) and its correlation with blood levels of KB, glucose, L-lactate, ammonia and urea.

To simulate an accelerated mobilization of free fatty acid from adipose tissue, as occurs in uncontrolled type 1 diabetes, livers were infused with a constant and saturating concentration of octanoate, i.e. the concentration in which the maximal liver KB production could be obtained. This experimental approach discards the influence of the variability of ketogenic substrates and allows comparison of the capacity to produce KB in livers from different metabolic conditions.[21]

Table 3 Liver basal production

	NDBT	DBT
Ketone bodies	0.16 ± 0.04	0.14 ± 0.03
Glucose	0.23 ± 0.06*	6.64 ± 0.98
L-lactate	0.54 ± 0.04*	1.47 ± 0.05
Urea	1.01 ± 0.32*	1.88 ± 0.13

Basal ketone bodies, glucose, L-lactate and urea production in the livers of 15 h fasted non-diabetic (NDBT) and diabetic (DBT) rats two hours after insulin administration. The area under curve values (μmol/g) obtained as described in Materials and methods were expressed as means ± SD of six to eight liver perfusion experiments

*$P < 0.05$ when compared with diabetic (DBT)

Table 4 Liver ketone bodies production from octanoate 0.3 mmol/L

NDBT	DBT
1.09 ± 0.23*	2.09 ± 0.37

Ketone bodies production from the saturating concentration of octanoate in the livers of non-diabetic (NDBT) and diabetic (DBT) rats two hours after intraperitoneal administration of Detemir insulin (1 U/kg) in 15 h fasted rats. The area under curve values (μmol/g) obtained as described in Materials and methods were expressed as means ± SD of six to eight liver perfusion experiments

*$P < 0.05$ when compared with diabetic (DBT)

Table 5 Liver production

	L-alanine		L-lactate		Pyruvate		Glycerol	
Liver production	**NDBT**	**DBT**	**NDBT**	**DBT**	**NDBT**	**DBT**	**NDBT**	**DBT**
Glucose	2.78 ± 0.48	ND	6.86 ± 0.72	ND	5.57 ± 0.73	ND	3.67 ± 0.25*	2.64 ± 0.47
Urea	2.40 ± 0.23*	4.97 ± 0.69	Nd	Nd	Nd	Nd	Nd	Nd
L-lactate	0.83 ± 0.19*	4.84 ± 0.46	Nd	Nd	15.65 ± 0.06*	10.63 ± 0.63	Nd	Nd
Pyruvate	0.39 ± 0.10*	5.52 ± 0.15	3.11 ± 0.55*	4.96 ± 0.23	Nd	Nd	Nd	Nd

ND: not detected. Nd: not determined

Liver production of glucose, urea, L-lactate and pyruvate from saturating concentration of L-alanine, L-lactate, pyruvate or glycerol in non-diabetic (NDBT) and diabetic (DBT) rats two hours after intraperitoneal administration of Detemir insulin (1 U/kg) in 15 h fasted rats. The area under curve values (μmol/g) obtained as described in Materials and methods were expressed as means ± SD of six to eight liver perfusion experiments. *$P < 0.05$ (NDBT versus DBT group)

The reason for using this medium-chain fatty acid was based on the following facts: high solubility in water; its transport through the mitochondrial membrane does not demand a carnitine-dependent transport mechanism; it is exclusively metabolized by the mitochondria; and its higher capacity to produce KB when compared with other fatty acids.[22,23]

As shown in Table 3, the basal ketogenesis (before octanoate infusion) from endogenous fatty acids was negligible. These results are in agreement with the classical view[1,5] that the plasma free fatty acid levels elevation is a *sine qua non* condition to occur in significant rates of ketogenesis. However, in spite of the fact that insulin inhibits ketogenesis,[24] livers from DBT rats submitted to long-term IIH showed higher ($P < 0.05$) KB production from octanoate (Table 4). This result could be explained, partly at least by a higher liver beta oxidation, meaning a tendency toward a more negative mitochondrial NADH:NAD^+ potential. In agreement with this suggestion, livers from DBT rats showed a higher ($P < 0.05$) β-hydroxy-butyrate:acetoacetate ratio (results not shown). Furthermore, the mechanisms involved in the increased capacity of producing KB from octanoate in the livers of rats submitted to long-term IIH probably involves an increased release of counterregulatory hormones,[9,24] which could overcome the inhibitory insulin effect on liver ketogenesis.

Additionally, the decreased blood KB levels after insulin administration in NDBT and DBT rats (Table 2) was probably due to the direct inhibitory effect of insulin in the transport of long-chain fatty acids across the mitochondrial membrane. But it must be emphasized that there is a rebound increase in blood KB concentration during the glucose recovery phase of IIH (from 4 h until 10 h after insulin administration) in DBT and NDBT rats (Table 2). The rebound increase was more intense in DBT rats probably due to the fact that alloxan DBT rats show very low availability of endogenous insulin and there is a prevalence of the effect of counterregulatory hormones[24,25] after the dissipation of the administered insulin.

A mixed acid–base disorder may be present in diabetic patients. The lactic acidosis is attributed to a peripheral circulatory failure that occurs during the diabetic ketoacidosis.[6] But our results suggest that the increased blood levels of L-lactate of DBT rats (Table 2) could be attributed, partly at least, to the higher liver production of L-lactate (Table 3).

Thus a question can be raised: how does one explain the elevated L-lactate production in the livers of DBT rats? To answer this question, it is necessary to consider that the livers of DBT rats showed higher glucose production from glycogenolysis (Table 3) and part of this glucose enters glycolysis, producing L-lactate.[26,27] In agreement with these reports, DBT rats showed higher ($P < 0.05$) liver basal production of L-lactate (Table 3) and higher ($P < 0.05$) blood levels of L-lactate (Table 2) in comparison with NDBT rats. In addition, the administration of insulin, which activates liver glycolysis, increased blood levels of L-lactate not only in DBT but also in NDBT rats (Table 2). However, the changes were more intense ($P < 0.05$) in the DBT group (DBT versus NDBT groups) in what could be interpreted as a higher predisposition to hyperlactemia.

Another remarkable feature of the experiments herein reported is that except for glycerol, a negligible gluconeogenesis was detected in the livers of DBT rats. A possible explanation for these results was the fact that when the rate of ammonia production is higher than its conversion to urea, the increased intramitochondrial concentration of ammonia decreased the intermediates of tricarboxylic acid leading to depletion of ATP and consequently an inhibition of gluconeogenesis.[20] In the specific case of L-alanine, its conversion to glucose includes a step inhibited by ammonia, i.e. the pyruvate carboxylation.[28] In agreement with this suggestion, DBT rats showed higher ($P < 0.05$) blood ammonia levels (Table 2) and increased catabolism of amino acids, inferred by the higher ($P < 0.05$) urea production from L-alanine (Table 5). In addition, the possibility that the glucose precursors that were not converted to glucose, particularly pyruvate, could be converted to L-lactate contributing to the higher blood levels of L-lactate of DBT rats must be considered (Table 2). On the other hand, the maintained liver capacity to produce glucose from glycerol in DBT rats probably reflects the fact that this glucose precursor bypasses the pyruvate carboxylase and phosphoenolpyruvate carboxykinase enzymes and enters into gluconeogenesis after the triose phosphate step. Furthermore, the lower gluconeogenic activities of DBT rats help us to understand the slower glucose recovery in DBT rats in comparison with NDBT rats (Table 2).

In summary, our previous results[12,13] and the data shown here reinforce the suggestion that during long-term IIH the increased liver ketogenic capacity, if associated with a free fatty acids elevation that occurs during the dissipation of insulin effects,[24,25] augmented the risk of hyperketonemia. Therefore, our results have great clinical interest because they help one to understand the exaggerated rates of ketogenesis during the recovery phase of hypoglycemia in

diabetic patients[7,8] and suggest a more rigid control of KB blood levels during the glucose recovery phase of IIH. Furthermore, in spite of the fact that the insulin injection decreases blood KB, it is necessary to consider the risk of worsening lactic acidosis.

However, future investigations are necessary to confirm whether these acute effects on blood levels of KB and lactic acid could occur during long-term insulin treatment in diabetic animals.

Author contributions: All authors participated in conducting the experiments, interpretation of the studies and analysis of the data. FPMS, HCB, VAFG and MMDPF designed experiments. FPMS, VAFG and RBB wrote the manuscript.

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