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Exercise partially reverses the inhibitory effect of caffeine on liver gluconeogenesis in type 1 diabetic rats with hypoglycemia

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The purpose was to determine the possible effects of exercise and/or caffeine on hypoglycemia and liver gluconeogenesis in diabetic rats. These were divided into four subgroups: (a) intraperitoneal insulin only, (b) exercise bout before insulin, (c) caffeine after insulin, and (d) exercise bout before and caffeine after insulin. The marked glycemic drop 45 min after insulin (0 min = 229.00, 45 min = 75.75) was considerably reduced ($p < 0.05$) by caffeine or exercise (45 min: exercise = 127.00, caffeine = 104.78). However, this systemic effect was lost ($p > 0.05$) when they were combined (45 min: exercise + caffeine = 65.44) (Mean, in $mg \cdot dL^{-1}$). Caffeine alone strongly inhibited liver glucose production from 2 mM lactate 45 min after insulin (without caffeine = 3.05, with caffeine = 0.27; $p < 0.05$), while exercise + caffeine partially re-established the liver gluconeogenic capacity (exercise + caffeine = 1.61; $p < 0.05$ relative to the other groups) (Mean, in $\mu mol \cdot g^{-1}$). The improved hypoglycemia with caffeine or exercise cannot be explained by their actions on liver gluconeogenesis. As their beneficial effect disappeared when they were combined, such association in diabetic patients should be avoided during the period of hyperinsulinemia due to the risk of severe hypoglycemia.

Keywords: exercise, caffeine, type 1 diabetes, insulin, rats, liver

Introduction

The maintenance of glycemia is a finely tuned mechanism that demands the integration of glucose absorption, production, and uptake (30), as well as the presence of signaling molecules such as insulin, glucagon, adrenaline, adenosine, and others (13). The liver is a multifunctional organ with an active role on this process, operating as a glycemic buffer by promoting synthesis/degradation of the glycogen store as well as synthesizing new glucose molecules (gluconeogenesis) (31). These metabolic pathways are constantly modulated by many factors, such as nutritional condition and physical activity (5).

The metabolic disorder resulting from lack of insulin is known as type 1 diabetes mellitus (T1DM) (11, 32, 39). It is characterized by an unbalanced increase in glycemia that demands the use of exogenous insulin to normalize the blood glucose levels and preserve homeostasis.

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Many T1DM patients are young and physically active, and exercise in T1DM can trigger adverse effects such as hypoglycemia and hyperglycemia. More than 30% of children and youngsters with T1DM show hypoglycemic episodes during an exercise session. On the other hand, hyperglycemia disrupts the metabolic and vascular benefits of exercise (20). Despite an intense and rigorous glycemic control preventing the secondary complications of diabetes, there is an intrinsic inability in regulating glucose concentration during and after exercise. In addition, nocturnal hypoglycemia is common in T1DM on the night following exercise and, as in a vicious cycle, the response to exercise on the following session is impaired (10).

There are many substances that interfere with the glycemic control, such as caffeine (1,3,7-trimethylxanthine), found in products widely consumed such as coffee, tea, guarana, chocolate, and medications (3, 16, 28, 47). Caffeine is avoided in some cases, such as autosomal dominant polycystic kidney disease, due to prior awareness of the patients of need for restriction, because caffeine may induce cyst and kidney enlargement (45). However, in other cases, in spite of not having nutritional value, caffeine has been used as an ergogenic substance because it improves performance in long-term physical activities. The ergogenic effect of caffeine is reversible and seems to be mediated by central and peripheral mechanisms of action that can trigger physiological and metabolic changes (19, 38, 40, 44). At first, the action of caffeine was linked to an increase in the serum concentration of adrenaline and noradrenaline, which would lead to the enhanced oxidation of free fatty acids and saving of glycogen by the muscle tissue (9, 19, 25, 38, 40, 44), but adenosine receptor antagonism seems to be the major mechanism of action of caffeine (4).

Adenosine is a ubiquitous cellular constituent released from the hydrolysis of adenosine triphosphate (ATP) in the extracellular space or intracellularly; the newly formed adenosine is exported to the extracellular space by specific nucleoside transporters (12, 22, 49). In the extracellular space, adenosine can bind to four different G-protein coupled receptors. The A1 and A3 receptors inhibit adenylyl cyclase, while the A2a and A2b receptors activate the enzyme (43). In the liver, the adenosine receptors can have many roles concerning glucose metabolism regulation, lipolysis, and inflammation (23, 37, 48). An increase in the expression of these receptors in the liver in adverse conditions like diabetes could link their activities with the pathogenesis of liver complications in T1DM (7, 15, 21, 29). In addition, situations that increase the levels of ATP hydrolysis, such as exercise and T1DM, are accompanied by increased levels of adenosine (10, 35). This study assessed the *in vivo* glycemic response of T1DM rats made hypoglycemic by insulin injection, as well as their liver gluconeogenic activity, in the presence of exercise, and/or caffeine. The purpose was to determine the possible synergistic or antagonistic effects of these agents on the drop and recovery of glycemia. Particularly, *in situ* liver perfusion assessed the liver contribution to the glycemic response observed. Exercise and caffeine both had a beneficial effect on the glycemic profile of T1DM rats during the hypoglycemic episode. However, when combined, these effects disappeared. In addition, the *in vivo* glycemic profile could not be readily explained by the observed liver gluconeogenesis, indicating that the systemic effects of exercise and caffeine are much more complex than their hepatic effects.

Materials and Methods

Materials

Regular human insulin (Novolin®) was obtained from Novo Nordisk (Brazil). Alloxan was obtained from Sigma (Brazil). All other chemicals used had high degree of purity.

Animals

All experimental procedures were approved by The University of Maringá Ethics Committee (CEAE no. 002/2010 and CEAE no. 069/2013). Male Wistar rats (*Rattus norvegicus*) weighing 180 g and aging 59 days were kept at the animal house of the Department of Physiological Sciences under controlled temperature (23 °C) and photoperiod (12 h light/12 h dark). Water and balanced Nuvital® chow were given freely until the day before the experiment, when the chow was removed, so that the animals had a 14 h fasting (overnight) at the beginning of the experimental protocols. For the *in vivo* experiments, the animals remained under fasting until the end of the protocols, when they were returned to their cages.

Diabetes induction

The animals were given an intravenous injection of alloxan (40 mg·kg·bw^{-1}). After 4 days, the glycemia during the fasted state was checked through the caudal vein using glucose test strips and a glucometer (Optium Xceed). All animals having fasting blood glucose values $\geq$200 mg·dL^{-1} were included in the study. The experimental protocols were carried out on the following day.

Experimental groups

For both the *in vivo* and *in situ* experiments, the type 1 diabetic animals (T1DM) were divided into four subgroups. Those receiving insulin injection (T1DM+I), those subjected to an acute exercise bout before insulin injection (T1DM+E+I), those given caffeine 15 min after insulin injection (T1DM+I+C), and those subjected to an acute exercise bout before and given caffeine 15 min after insulin injection (T1DM+E+I+C).

Exercise bout

The exercise bout (groups T1DM+E+I and T1DM+E+I+C) consisted of a run in a programmed treadmill (KT3000, Ibramed, Brazil), adapted for training rats. The animals did not have an adaptation period before the experiments. Initial treadmill speed was 0.5 km·h^{-1}, with progressive increases of 0.2 km·h^{-1} every 2 min, until exhaustion, characterizing an acute exercise bout. Exhaustion was determined through visual observation and defined as the moment when the animal could not keep up with the treadmill speed. Insulin was given immediately after exhaustion.

Insulin-induced hypoglycemia (IIH)

Hypoglycemia was induced in all the groups through intraperitoneal (ip.) injection of regular, fast-acting insulin (1 U·kg·bw^{-1}). The glycemia of the animals was recorded through the caudal vein using glucose test strips and a glucometer (Optium Xceed) during 5 h after insulin injection (times 0, 15, 30, 45, 60, 120, 180, and 300 min). The values were expressed as mg·dL^{-1}.

Caffeine administration

Caffeine was administered orally (gavage) at a dose of 5 mg·kg·bw^{-1} 15 min after the ip. injection of regular insulin (T1DM+I+C and T1DM+E+I+C), while the other groups were given an equivalent volume of saline.

In situ *liver perfusion*

The perfusion system is composed of vessels for the perfusion fluid, a peristaltic pump and a membrane oxygenator, coupled to a water bath with external circulation of warm water and a

cylinder of carbogenic mixture ($O_2/CO_2 = 95/5\%$). The perfusion fluid used was Krebs-Henseleit-bicarbonate (KH) with pH 7.4 at 37 °C. Forty-five minutes after insulin injection (the time of the lowest glycemia of the T1DM+I group, see Fig. 2), animals were ip. anesthetized with sodium pentobarbital (40 mg·kg·bw^{-1}) and subjected to laparotomy. The portal vein was cannulated under low flux (1 mL·min^{-1}·g^{-1}) and another cannula was inserted into the inferior cava vein to allow the rapid exsanguination of the liver while the flux was elevated to about 4 mL·min^{-1}·g^{-1} to assure an adequate supply of oxygen to the organ. After 30 min of perfusion for the stabilization of the preparation, samples of the effluent fluid were collected each 5 min for further biochemical assays. During the first 10 min of collection, the perfusion was made with KH alone and then L-lactate was added to the KH as gluconeogenic precursor for 20 min, at a concentration of 2 mM, a near-physiological blood value during fasting (2).

Demonstrative experiment for the calculation of the area under the curve

After a perfusion period of 10 min (basal glucose production), L-lactate was dissolved in the perfusion fluid and perfused from the 10th to the 30th min. Samples of the effluent fluid were collected at 5-min intervals and the glucose concentrations were determined (6). The difference in the glucose production during the perfusion with L-lactate and the basal glucose production was used to calculate the area under the curve (AUC), whose value was expressed in μmol·g^{-1} liver (Fig. 1). The AUCs in Fig. 3 and Table I were obtained from similar procedures.

Determination of glucose and pyruvate in the perfusate

Glucose was measured by the glucose-oxidase method (6) and pyruvate by a previously described technique (8). The values of glucose and pyruvate in the perfusate samples were expressed in μmol·min^{-1}·g^{-1} liver.

Statistical Analysis

The results [mean ± standard deviation (SD) of 6–10 experiments per group] were assessed through student's *t*-test, using the T1DM+I as the reference group, with the software GraphPad Prism 5.0. The significance level was set at 5%.

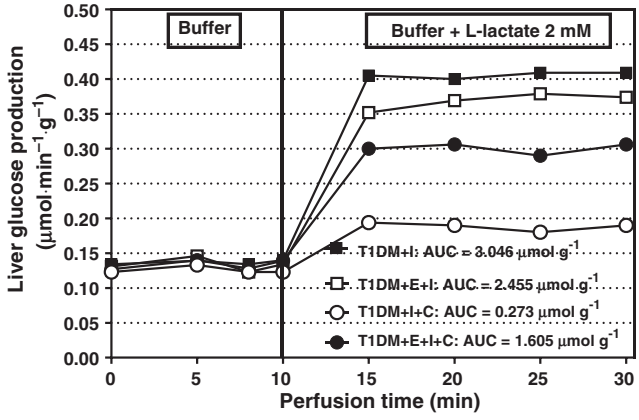

Fig. 1. Demonstrative experiment of the glucose production (μmol·min^{-1}·g^{-1}) from 2 mM L-lactate in liver perfusion of T1DM+I, T1DM+E+I, T1DM+I+C, and T1DM+E+I+C rats. The data are of one animal of each group

Table I. Liver pyruvate production from 2 mM L-lactate in T1DM+I, T1DM+E+I, T1DM+I+C, and T1DM+E+I+C rats. The AUCs are expressed in μmol·g^{-1} liver as mean ± SD of six to eight perfusion experiments

Group	Pyruvate production (μmol·g^{-1})
T1DM+I	4.97 ± 0.38
T1DM+E+I	6.68 ± 0.39*
T1DM+I+C	6.06 ± 0.43*
T1DM+E+I+C	4.86 ± 0.30

*$p < 0.05$ vs. T1DM+I

Results

The values of the fasting glycemia were similar across all the experimental groups. The T1DM animals previously subjected to exercise had a tendency of having a higher glycemia than those non-exercised; however, differences were not statistically significant (time 0 min, Fig. 2). In these same groups, the pre- and post-exercise glycemia were not statistically different either ($p > 0.05$ not shown). The time to exhaustion was similar in all the exercised groups and was of 8 min 20 s on average.

Figure 2 shows that soon after insulin injection hypoglycemia was established in all groups and reached the lowest values within the first 45 min. The diabetic group (T1DM+I) did not recover glycemia until time 300 min. However, exercise prior to insulin injection reduced the intensity of the hypoglycemic episode since time 15 min. The administration of caffeine resulted in a better hypoglycemic profile at times 30 and 45 min and better glycemia recovery at 300 min. On the other hand, the association of exercise and caffeine (T1DM+E+I+C) resulted in a severe hypoglycemic crisis after 45 min, similar to the group given only insulin (T1DM+I). Nevertheless, it did not interfere with the capacity of glycemia recovery observed at 300 min. Therefore, exercise, caffeine, and the combination of both promoted a significant recovery at 300 min.

Considering that the hypoglycemic profile was attenuated by either exercise or caffeine, but not their association, a possible effect of the treatments on the liver glucose metabolism was investigated through the *in situ* liver perfusion. As the lowest glycemic values were found 45 min after insulin injection, this time was chosen for the liver experiments (Fig. 3, Table I).

As for the liver glucose production from L-lactate (Fig. 3), it was observed that the previous exercise bout did not change this pathway ($p > 0.05$). However, liver

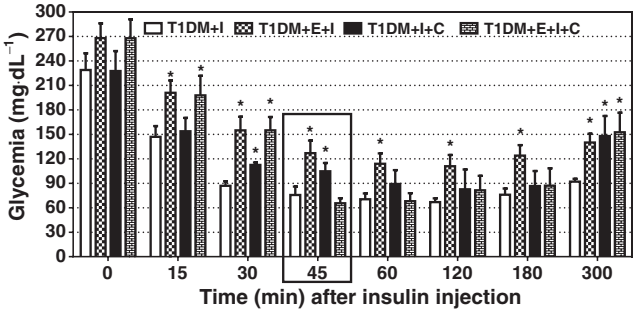

Fig. 2. Glycemic response during IIH. The bars represent the mean ± SD of six to eight experiments. *$p < 0.05$ vs. T1DM+I at each time

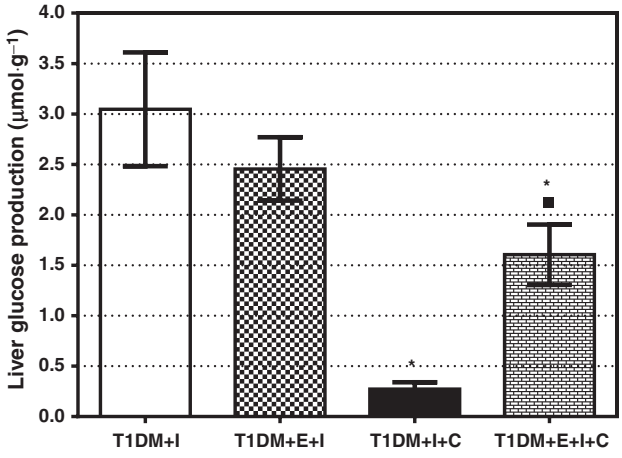

Fig. 3. Liver glucose production from 2 mM L-lactate in T1DM+I, T1DM+E+I, T1DM+I+C, and T1DM+E+I+C rats. The AUCs are expressed in μmol·g^{-1} liver as mean ± SD of six to eight perfusion experiments. *$p < 0.05$ vs. T1DM+I, ▪$p < 0.05$ vs. T1DM+I+C

gluconeogenesis was about 10 times lower in the presence of caffeine. Importantly, it was also observed that the association of exercise and caffeine partially re-established the liver capacity of producing glucose from lactate.

As L-lactate is oxidized to pyruvate to be converted to glucose, the liver production of pyruvate from L-lactate was also measured in the perfused liver (Table I). It was observed that in the presence of exercise or caffeine, there were higher pyruvate values than on the other treatments.

Discussion

The *in vivo* results showed that insulin-injected T1DM rats had a hypoglycemic episode that did not recover completely after 300 min (Fig. 2, group T1DM+I).

Since exercise and insulin alone cause an amplifying effect on the number of active GLUT4, which intensifies the glucose use by the skeletal muscle cells (33, 34), it was expected that their combination would worsen the hypoglycemia. However, the association of insulin and exercise (Fig. 2, group T1DM+E+I) prevented the lowering of the glycemia to the values of the T1DM+I group. This observation can be explained by the fact that exercise also increases the release of catecholamines, which cause liver gluconeogenesis and glucose release and stimulates lipolysis and the release of free fatty acids in the adipose tissue (17). This scenario demonstrates that exercise could prevent a more severe hypoglycemia and have an important role in the glycemic control in T1DM.

On the other hand, caffeine caused a less pronounced drop of the glycemic level at 30, 45, and 300 min (group T1DM+I+C, Fig. 2), but it was not different from the T1DM+I group from 60 to 180 min, i.e., caffeine demonstrated a biphasic effect, an acute effect at 30 and 45 min (higher blood insulin), and a long-term effect at 300 min (after blood insulin dissipation). The antagonistic effects of caffeine on the adenosine receptors are its major mechanism of action (4). These receptors are found in muscle, liver, and adipose tissue, all of them heavily involved in blood glucose homeostasis. In adipose tissue (A1-subtype receptors), caffeine could result in increased lipolysis and a glucose-saving systemic effect (3, 19, 24, 25, 27, 38); however, by antagonizing adenosine receptors on skeletal muscle (also A1-subtype receptors), caffeine increases glycolysis (27). In addition, adenosine receptors in

the liver stimulate gluconeogenesis, glycogenolysis, and glucose release (14, 15, 18, 21, 27, 36), all of which could be potentially antagonized by caffeine.

Although caffeine also stimulates the release of catecholamines, the dose should be at least 20 times greater than that used in these experiments (47). It seems that at the dose of caffeine employed (5 mg·kg·bw^{-1}), there is a predominance of the antagonistic effect on the adenosine receptors. This adenosine antagonism by caffeine is the sum of all its effects on many tissues, and may be dependent, among other things, on the relative amount of muscle – resulting in glucose consumption – and fat – resulting in glucose sparing (25), in addition to its effects on the liver.

The possibilities listed could result in the less severe hypoglycemic profile of the T1DM+E+I and T1DM+I+C groups. Within this reasoning, we can argue that exercise and caffeine efficiently antagonized hypoglycemia and decreased the glycemic drop, especially during the first 45 min after insulin injection, leading to a less marked hypoglycemic profile.

On the other hand, the association of exercise and caffeine (Fig. 2, T1DM+E+I+C) caused a glycemic drop similar to insulin (group T1DM+I) between 45 and 180 min. In this case, caffeine may have progressively inhibited the effect of exercise on the muscle cells, so that from 45 to 180 min, the anti-hypoglycemic effects of exercise were completely overcome by caffeine. This may have been caused by caffeine competitively inhibiting the binding of extracellular adenosine with its membrane receptors, which would tend to increase glycolysis and glucose uptake in muscle cells and decrease glucose production and release from the liver (10, 17, 24, 35, 42).

However, both exercise and caffeine, either isolated or associated, had a residual effect and markedly recovered the glycemia after insulin disappearance (Fig. 2, 300 min), probably but not exclusively due to an increased lipolysis powered by caffeine and exercise-derived catecholamines (26, 38, 41, 46). It seems that both exercise and/or caffeine are effectively acting as long-term counter-regulatory mechanisms, as judged from the improved glycemia (compared to group T1DM+I) 300 min after insulin injection.

In non-diabetic animals, differently from the T1DM animals, the hypoglycemic profile is not changed by either exercise or caffeine; instead, their association results in a better hypoglycemic profile (4). This difference is most certainly due to the diabetic condition of the animals in this study, because diabetes changes the signaling capacities of many substances (such as exercise-derived catecholamines and adenosine) on the major tissues involved in the response to hypoglycemic conditions, i.e., skeletal muscle, liver, and adipose tissue.

The conversion of L-lactate to pyruvate is the crucial step for L-lactate to be used as gluconeogenic precursor. Liver pyruvate production (Table I) had a qualitative resemblance to the *in vivo* glycemic profile of the groups 45 min after insulin injection: lower values in the T1DM+I and T1DM+E+I+C groups and higher values in the T1DM+E+I and T1DM+I+C groups. Although the groups have shown increased production of pyruvate, the production of glucose was reduced in the groups T1DM+I+C and T1DM+E+I+C (Fig. 3). It is possible that, in these groups, pyruvate was being diverted to some other cellular pathway, such as that of nitrogen rescue. Therefore, further studies are needed to substantiate these results.

Exercise (T1DM+E+I) did not significantly change the liver gluconeogenic capacity from L-lactate during hypoglycemia when compared to the T1DM+I group (Fig. 3). As the hypoglycemic profile of these two groups were significantly different at 45 min of the IIH – the time chosen to assess liver gluconeogenesis – the liver glucose production from L-lactate cannot account for the difference observed *in vivo*. In this case, the extra-hepatic effects of exercise discussed above predominate over the liver effects, and manifest as variation in blood glucose levels.

On the other hand, caffeine (T1DM+I+C) caused a marked reduction of the liver glucose production from L-lactate. It is possible that caffeine efficiently blocked the liver adenosine receptors (7, 44), making way to an enhancement of the inhibitory effect of insulin on the liver glucose production. This suggestion is consistent with the inhibition of A3-subtype receptors (8, 29) and with the results of the *in situ* liver perfusion for the T1DM+I+C group (Fig. 3). However, as hypoglycemia was less severe with caffeine 45 min after insulin injection, extra-hepatic effects were probably predominant. When exercise and caffeine were associated (group T1DM+E+I+C, Fig. 3), liver glucose production was partially restored. Exercise promotes catecholamine release and increased levels of endogenous adenosine (1, 5, 10), both of which could have partially overcome the inhibitory effect of caffeine on liver gluconeogenesis from L-lactate.

Conclusions

In summary, the correlation of the liver glucose production from L-lactate with the hypoglycemic profile of the diabetic animals shows that the systemic effects of exercise and caffeine are much more complex than their hepatic effects, so that the *in vivo* glycemic profile cannot be readily explained by the observed liver gluconeogenesis. This suggests that the systemic effects of exercise and caffeine on the glycemic levels are also dependent upon their actions on other important targets, such as skeletal muscle and adipose tissue. Finally, both exercise and caffeine have a beneficial effect on the glycemic profile of T1DM rats during the decay phase hypoglycemic episode. However, when combined, these effects disappear and therefore the association of exercise and caffeine in T1DM should be avoided during the period of hyperinsulinemia due to the risk of severe hypoglycemia.

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