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Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: effects on hepatic glucose release and glycaemia

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The effects of chlorogenic acid (CA) on hepatic glucose output, blood glucose levels and on glucose tolerance were analysed. Hepatic uptake of CA and its effects on hepatic catabolism of L-alanine and glucose-6-phosphatase (G-6-Pase) activity were also evaluated. CA (1 mM) inhibited about 40% of G-6-Pase activity ($p < 0.05$) in the microsomal fraction of hepatocytes, but no effect was observed on production of glucose from gluconeogenesis or on L-alanine catabolism, at various concentrations of CA (0.33, 0.5 and 1 mM), in liver perfusion experiments. Since there were indications of a lack of uptake of CA by the liver, it is possible that this compound did not reach sufficiently high intracellular levels to inhibit the target enzyme. Accordingly, intravenous administration of CA also failed to provoke a reduction in blood glucose levels. However, CA did promote a significant reduction ($p < 0.05$) in the plasma glucose peak at 10 and 15 min during the oral glucose tolerance test, probably by attenuating intestinal glucose absorption, suggesting a possible role for it as a glycaemic index lowering agent and highlighting it as a compound of interest for reducing the risk of developing type 2 diabetes.

KEY WORDS — chlorogenic acid; glucose-6-phosphatase; gluconeogenesis; liver perfusion; hepatic glucose release; glycaemia; glycaemic index; glucose tolerance test

INTRODUCTION

Chlorogenic acid (CA) is a natural phenolic compound found in a variety of foodstuffs, of which coffee is the main source in the human diet. This compound and some synthetic derivatives have been shown to inhibit glucose-6-phosphatase (G-6-Pase), the enzyme that catalyzes the final step of glycogenolysis and gluconeogenesis, which are the two main metabolic pathways responsible for the release of glucose in the liver.[1–7] Furthermore, it has been demonstrated that by inhibiting G-6-Pase, these derivatives of CA lower the rate of hepatic release of glucose arising from these two pathways,[4–6,8–11] as well as decreasing the blood glucose concentration,[4,5,12,13] thus offering an important possible means of regulating the overproduction of glucose in the liver[14] and of controlling glycaemia in type 2 diabetics.

On the other hand, some research indicates that CA inhibits glucose transport through the epithelial cells of the intestine. Thus, it reduced by 80% the capacity for transport of glucose in brush-border membrane vesicles isolated from the small intestine of rats.[15] Interestingly, there have also been reports that high doses of coffee promote a slight fall in glycaemia in

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both humans and rats[16–18] and that regular consumption of coffee diminishes the risk of developing type 2 diabetes.[16,19–22] In support of these observations, current reviews describe CA as potentially lowering the glycaemic index of food, and is the component of coffee considered responsible for inhibiting, and hence slowing, glucose uptake in the small intestine.[23–25] Since there are no conclusive data in the literature on the influence of the natural compound CA on glucose output from the liver, blood glucose levels or on intestinal absorption of glucose, these effects of CA were analysed in this study. In addition, the uptake of CA by the liver and its effects on hepatic catabolism of L-alanine were investigated.

MATERIALS AND METHODS

Animals

Male albino Wistar rats, body weight (bw) 170–200 g, were housed in a constant 12/12 h light/dark cycle at $23 \pm 2^{\circ}C$ and supplied freely with water and a standard commercial laboratory diet (Nuvilab®). Animals were fed or fasted for 24 h before the experiments. All experiments started at 2 p.m. to minimize circadian variation. The experimental protocols were executed in accordance with Brazilian law and approved by the Ethics Committee of the State University of Londrina.

Chemicals

CA (5-caffeoylquinic acid) was obtained from Sigma Chemical Co. (St. Louis, USA). Gluconeogenic precursors and all other chemicals were of the best available grade (98–99.8% purity) and were purchased from Acros Organics (New Jersey, USA), Reagen (Rio de Janeiro, Brazil), Sigma Chemical Co. (St. Louis, USA) and Merck (Darmstad, Germany).

Microsome isolation

Intact microsomes were obtained from livers of rats fasted for 24 h, by differential centrifugation, following a previously described method,[26–30] with modifications. The microsomal fraction was isolated by homogenizing the liver in 10 volumes of buffer (1.15% KCl, 10 mM Tris, 100 μM phenylmethylsulfonyl fluoride; pH 7.4), in a Dounce tissue grinder chilled in ice. The homogenate was filtered through a fine gauze and centrifuged, in sequence, at 2550, 7080 and 12434*g*, for 10 min each time. The final supernatant was ultracentrifuged at 105 000*g* for 1 h and the resulting sediment resuspended in the same buffer, at 30 mg protein per ml. This microsome suspension was used to assay G-6-Pase in the absence (control) and presence of CA.

Liver perfusion

Livers of rats fasted for 24 h were perfused *in situ* in a non-recirculating system, using haemoglobin-free medium and monovascular anterograde liver perfusion.[31–32] The animals were first anaesthetized with sodium pentobarbital ($40\,mg\,kg^{-1}$) and subjected to laparotomy. The perfusion fluid, Krebs-Henseleit buffer (KHB), pH 7.4, at 37°C and saturated with a 95%:5% O_2:CO_2 mixture, was introduced through a cannula inserted into the hepatic portal vein, while a second cannula in the inferior vena cava was used to collect the perfusate flowing out from the hepatic vein. In each individual experiment, the flow rate was corrected according to the weight of the liver: $4\,ml\,min^{-1}$ per gram fresh weight. To assess the influence of CA on hepatic glucose-related metabolism, the liver was perfused for the first 10 min with KHB alone and subsequently with KHB containing a gluconeogenic precursor (2.5 mM L-alanine, 2 mM glycerol or 2 mM L-lactate), with or without the addition of CA (0.33, 0.5 or 1 mM). During the perfusion, samples of perfusate were collected at intervals of 2 min, to measure their metabolite contents. At the end of the perfusion, the liver was removed and weighed, to allow precise metabolic calculations and the correction of flow rates. The program GraphPad Prism 4.0 was used to calculate the areas under the curves (AUCs).

Uptake of chlorogenic acid by the liver

CA uptake by hepatocytes was estimated by perfusing the liver with 0.33, 0.5 or 1.0 mM CA dissolved in KHB and measuring its concentration in the perfusion fluid, before entering and after leaving the liver.

Intravenous administration of chlorogenic acid

A solution of CA in 5 mM phosphate buffer (pH 7.4) was injected intravenously, at $70\,mg\,kg^{-1}$ bw, into fed rats that had been anaesthetized with ethyl ether. Blood samples for glucose determination were collected by decapitation at 5, 15, 30 and 60 min after the injection. Control rats were treated identically, except that buffer without CA was injected.

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Oral glucose tolerance test

In the pretreatment, CA solution (3.5 mg kg^{-1} bw) or the same volume of water (control group) were given by mouth to rats that had been deprived of food (for 24 h) and water (for 2 h). After 10 min, a solution of glucose (200 mg kg^{-1} bw) was given orally, with CA (3.5 mg kg^{-1} bw) or without CA (control rats). Blood samples were collected from the tip of the tail before (0 min) and 5, 10, 15, 30, 45, 60 and 90 min after administration of the glucose solution, to assay the blood glucose level. Since the pH of the glucose solution with CA was 2.8, while that of the control solution was 6.2, further control tests were carried out, in which the glucose solution was acidified with HCl to pH 2.8, to observe any effects of pH on the glucose tolerance test results.

Metabolite assays

Microsomal G-6-Pase activity was determined from the rate of release of phosphate under the assay conditions described originally by Fiske and Subbarow [33]. The microsomal suspension was incubated, at 37°C, in reaction medium (100 mM KCl, 20 mM Tris, pH 6.5), with 0 (control), 0.25, 0.5, 0.75 or 1.0 mM CA. The reaction was started by adding the substrate G-6-P at 10 mM and stopped by adding trichloracetic acid to precipitate the proteins. The released phosphate was analysed in the supernatant, after first buffering to pH 6.5 with sodium acetate, by a colorimetric reaction with ammonium molybdate in the presence of ascorbic acid. Protein contents of the ultracentrifuge supernatant fractions of liver homogenate were estimated by the Lowry *et al.*[34] method and the enzyme activity was expressed as micromoles phosphate released per minute per milligram protein.

Pyruvate[35] and urea[36] in the liver effluent perfusion fluid were assayed by standard enzymic methods. Since CA in the assay mixture can interfere with the glucose-oxidase reaction, glucose in samples of liver perfusate or blood plasma was determined by the *o*-toluidine method.[37] However, in the oral glucose tolerance test, glucose was analysed by the Accu-Chek Advantage II system.[38] This allowed blood glucose to be determined at frequent intervals (0, 5, 10, 15, 30, 45, 60 and 90 min) in the same rat, without anaesthetic, which can influence the response in the tolerance test.

CA was measured by high-performance liquid chromatography, with a diode-array detector (HPLC-DAD), in conditions described by Ky *et al.*[39]: A C18 column of 250 × 4 mm i.d. was packed with Merck Superspher; and used at a temperature of 30°C and flow-rate 0.8 ml min^{-1}. Elution was programmed with solvents A (2 mM phosphoric acid, pH 2.7, with 5% methanol v/v) and B (methanol with 5% of 2 mM phosphoric acid, pH 3.9); a linear gradient was used, starting with a 3:1 mixture of A:B and reaching pure B after 45min. Chromatograms were recorded with a DAD at 325 nm.

Statistical analysis

Normal distribution and variance homogeneity were tested and the appropriate statistical test (Student's *t*-test, Wilcoxon's test or ANOVA followed by Tukey) was employed to analyse the results, as indicated in the legends to the figures and Table 1. Statistical analysis was carried out with the program STATISTICA 5.1, at the 5% level of significance. Data are expressed as mean ± SEM.

RESULTS

At concentrations of 0.5, 0.75 and 1.0 mM, CA in the assay mixture inhibited hepatic G-6-Pase significantly ($p < 0.05$), and at 0.25 mM it showed an inhibitory tendency ($p > 0.05$). Over the whole range (0–1 mM), the effect was clearly dose-dependent and, at 1.0 mM, the inhibition reached about 40% (Figure 1).

Table 1. Chlorogenic acid (CA) concentrations before and after passing through liver

	CA after passing through liver		
CA before entering liver	10 min	30 min	50 min
59.3 ± 3.6 (0.33 mM) (3)	61.5 ± 3.5 (3)	—	—
96.8 ± 9.1 (0.5 mM) (3)	94.4 ± 3.0 (3)	—	—
261.8 ± 11.3 (1 mM) (3)	259.0 ± 15.1 (3)	277.0 ± 4.8 (3)	267.8 ± 3.8 (3)

Several concentrations (0.33, 0.5 or 1 mM) of CA were infused into the liver and samples of the effluent perfusate (having passed through liver) were collected after 10, 30 or 50 min to determine CA concentration (μg/ml).
Data reported as mean ± SEM. Results analysed by Student's *t*-test for paired samples and confirmed by the non-parametric Wilcoxon test, when necessary. () number of experiments.

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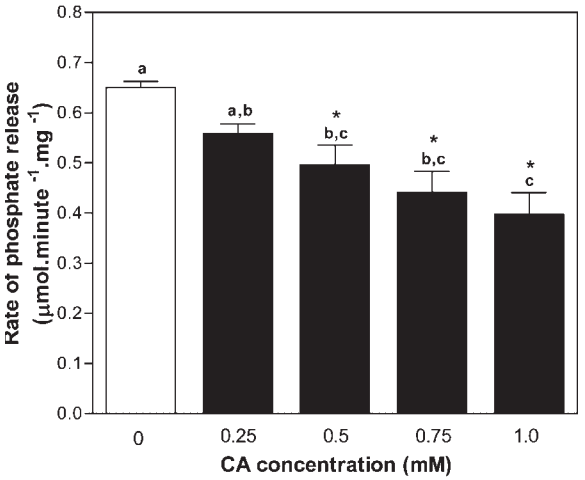

Figure 1. Effect of chlorogenic acid (CA) on G-6-Pase activity. Livers from 24 h fasted rats were homogenized and submitted to differential centrifugation as described in Methods. The microsomal fraction was used to assay G-6-Pase activity in the absence (□) or presence (■) of various concentrations of CA. Each bar represents the mean ± SEM of five determinations. Results were analysed by ANOVA, followed by a Tukey test. Distinct letters represent significant differences ($p < 0.05$). $^{*}p < 0.05$ versus control

In the liver perfusion experiments, low basal production rates were recorded for glucose (Figures 2A, 3A and 4A), pyruvate (Figure 2B) and urea (Figure 2C) in the initial 10 min of perfusion with KHB alone. Infusion of gluconeogenic precursors (L-alanine, glycerol or L-lactate) led to a progressive rise in production of the above metabolites (Figures 2–4). The addition of 0.33 mM CA to the perfusion fluid with L-alanine had no significant effect ($p > 0.5$) on the hepatic production of glucose (Figure 2A), pyruvate (Figure 2B) or urea (Figure 2C) from this precursor, as the respective AUCs confirm (Figure 2D): 4.35 ± 0.55, 4.64 ± 0.66, 4.44 ± 1.31 $\mu mol \cdot g^{-1}$ with CA, against 4.43 ± 0.31, 4.61 ± 0.56, 5.58 ± 1.12 $\mu mol \cdot g^{-1}$ without. Moreover, synthesis of glucose from glycerol was unaltered by 0.5 mM CA in the perfusion fluid ($p > 0.85$), the AUC being 6.17 ± 0.93 $\mu mol \cdot g^{-1}$ in the CA test and 6.33 ± 0.75 $\mu mol \cdot g^{-1}$ in the control (Figure 3A,B). Even when 1.0 mM CA was infused with precursor, the hepatic production of glucose from L-lactate was not affected ($p > 0.1$): AUC (CA) $= 1.96 \pm 0.28$ $\mu mol \cdot g^{-1}$, AUC (control) $= 1.34 \pm 0.44$ $\mu mol \cdot g^{-1}$ (Figure 4A,B). In congruity with this lack of any metabolic effect in liver perfusion experiments, no difference ($p > 0.3$) was detected between the concentrations of CA infused into the liver and those flowing out through the hepatic vein (Table 1), suggesting that uptake of CA in the liver was negligible.

Intravenous administration of CA (70 mg kg^{-1} bw) did not affect the glycaemia ($p > 0.15$) in rats sacrificed 5, 15, 30 or 60 min after the injection (Figure 5). In the oral glucose tolerance test, glycaemia started to rise 5 min after ingestion of glucose and reached peak values at 10–15 min, before falling back to near basal values by 90 min, in both control rats and those given CA by mouth. However, oral administration of CA (3.5 mg kg^{-1} bw) reduced the height of the glycaemic peak ($p < 0.05$) observed at 10–15 min in this test (Figure 6A): at 10 and 15 min, respectively, the control group had blood glucose levels of 113.8 ± 3.44 and 116.4 ± 2.73 mg dl^{-1}, while the group tested with CA had 102.25 ± 3.57 and 106.0 ± 2.34 mg dl^{-1}. Hence, the rise in glycaemia that followed the oral load of glucose was 21.8% and 17.8% lower, at 10 and 15 min, respectively, in animals that ingested CA (Figure 6B). As can be seen in Figure 6C, the reduction in pH of the glucose solution (to 2.8), caused by the addition of CA, did not alter the glycaemic peak ($p > 0.10$) in the oral glucose tolerance test.

DISCUSSION

G-6-Pase, a membrane-bound enzyme located in the endoplasmic reticulum of hepatocytes, catalyzes the breakdown of G-6-P to glucose and phosphate. This is an essential step in the release of glucose by the liver, performing a key role in the regulation of glycaemia.[3,4,7] *In vitro* tests with intact vesicles from hepatocyte endoplasmic reticulum have demonstrated that CA[1,3,6] and synthetic analogues of CA[2,5,40–42] inhibit the G-6-P transporter (T1) of the G-6-Pase enzyme complex and thus inhibit hydrolysis of G-6-P. The present tests with the liver microsome fraction confirm the inhibitory effect of CA on G-6-Pase activity *in vitro*. In Figure 1, it is clear that CA diminished G-6-Pase activity in a dose-dependent way, as did its synthetic derivatives.[6] The observed inhibition was about 40% at 1 mM CA and only a tendency at 0.25 mM. Nonetheless, in intact microsome preparations, 50% inhibition of this activity by 0.25 mM CA has been reported.[6]

Since a series of studies has demonstrated that CA synthetic analogues, by inhibiting G-6-Pase, can reduce the hepatic release of glucose arising from both glycogenolysis[3,5,9–10] and gluconeogenesis,[3,4,9–11] leading to a fall in glycaemia,[3,5,7,11–13]

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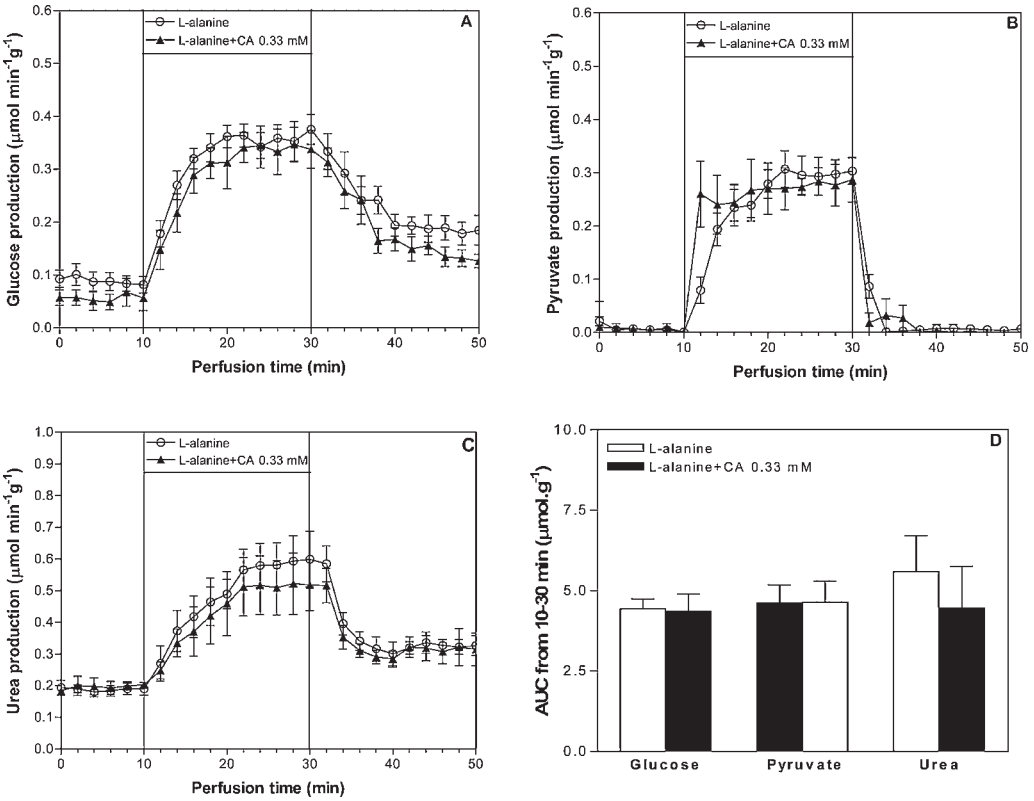

Figure 2. Effect of 0.33 mM chlorogenic acid (CA) on hepatic production of: (A) glucose, (B) pyruvate and (C) urea from L-alanine and (D) respective areas under the curve (AUC). Livers from 24 h fasted rats were submitted to *in situ* perfusion with Krebs-Henseleit buffer. 2.5 mM L-alanine (○) or 2.5 mM L-alanine + 0.33 mM CA (▲) were infused from 10 to 30 min. Each point represents the mean ± SEM of four or five experiments. Differences between the AUCs of control and experimental groups were analysed by Student's *t*-test for independent samples

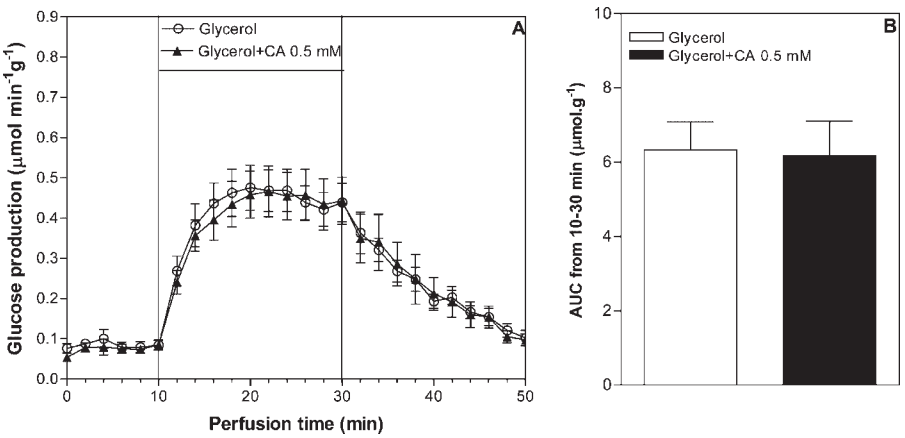

Figure 3. (A) Effect of 0.5 mM chlorogenic acid (CA) on hepatic production of glucose from glycerol and (B) respective areas under the curve (AUC). Livers from 24 h fasted rats were submitted to *in situ* perfusion with Krebs–Henseleit buffer. 2 mM glycerol (○) or 2 mM glycerol + 0.5 mM CA (▲) were infused from 10 to 30 min. Each point represents the mean ± SEM of four or five experiments. Differences between the AUCs of control and experimental groups were analysed by Student's *t*-test for independent samples

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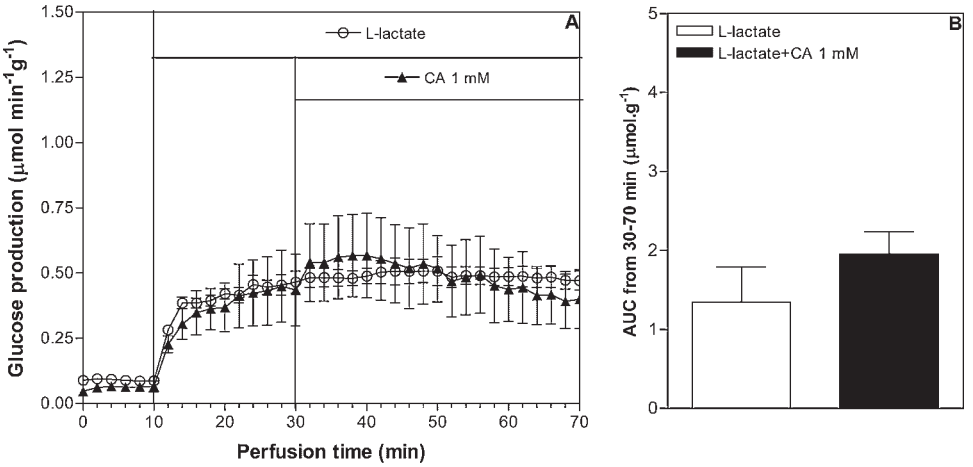

Figure 4. (A) Effect of 1 mM chlorogenic acid (CA) on hepatic production of glucose from L-lactate and (B) respective areas under the curve (AUC). Livers from 24 h fasted rats were submitted to *in situ* perfusion with Krebs–Henseleit buffer. Two millimolar L-lactate was infused from 10 to 30 min and 2 mM L-lactate (○) or 2 mM L-lactate + 1 mM CA (▲) were infused from 30 to 70 min. Each point represents the mean ± SEM of four to six experiments. Differences between the AUCs of control and experimental groups were analysed by Student's *t*-test for independent samples

we decided to investigate whether CA itself, the natural compound found in coffee and other foodstuffs, which is also the most specific inhibitor of the T1 unit of G-6-Pase,[3,6] possesses the same functional properties as its derivatives. Despite the inhibitory effect of CA on G-6-Pase *in vitro*, the results of liver perfusion studies showed that the same concentrations of CA (0.33, 0.5 and 1.0 mM) did not change the hepatic rate of production of glucose from gluconeogenetic precursors (Figures 2–4). Consistent with this, perfusion with 0.33 mM CA had no effect on the catabolism of L-alanine in the liver, as evidenced by formation rates of pyruvate and urea (Figure 2B,C); nor was the glycaemia altered by intravenous administration of a high dose (70 mg kg^{-1} bw) of CA (Figure 5).

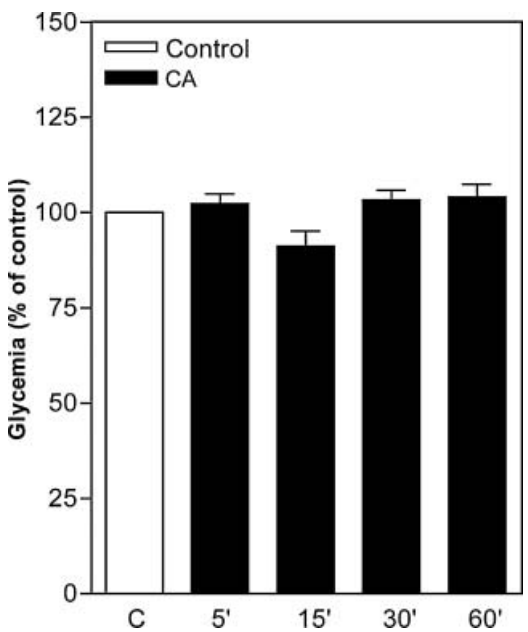

Figure 5. Effect of chlorogenic acid (CA) on glycaemia in fed rats. The glycaemia was measured 5, 15, 30 and 60 min after intravenous administration of CA (70 mg kg^{-1} bw) or phosphate buffer (control). Data are reported as % of control (C). Each bar represents the mean ± SEM of 6–10 experiments. Results, within a fixed time, were analysed by Student's *t*-test for independent samples

Glucose production by gluconeogenesis and glycogenolysis is inhibited in rats by liver perfusion with the CA derivatives S-4048,[10] S-3483[4] and compound 29i,[3] at concentrations similar to those used in this study, or even lower (S-3483, 0.1 mM).[4] In contrast to our findings with 70 mg kg^{-1} bw CA, glycaemia has been seen to fall after the administration of similar or lower (50 mg kg^{-1} bw) doses of CA derivatives.[13] IP or IV injection of S-4048 and IV infusion of S-4048[5,11–13] or S-3483[4,7] led to a reduction in glycaemia in both fed and fasted rats. Furthermore, IV infusion of S-3483[4] and S-4048[12] in fed rats prevented the hyperglycaemic peak induced by administration of glucagon.

The lack of effect of CA on either glucose release by the liver or glycaemia could be a result of low rates of uptake of CA, recently demonstrated,[43] or the lack of uptake by hepatocytes suggested by our liver perfusion results (Table 1). It has been proposed[43] that CA, unlike its derivatives that are carried through

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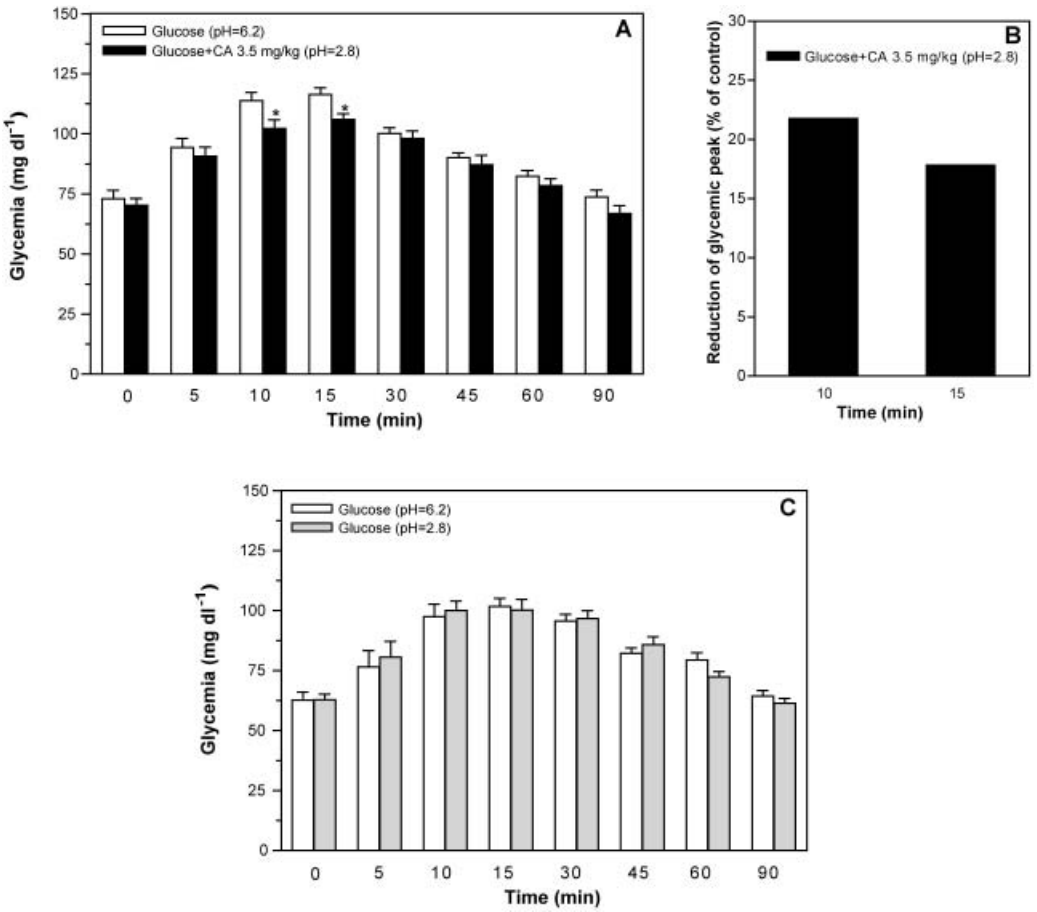

Figure 6. Effect of (A) chlorogenic acid (CA) and (C) pH on the oral glucose tolerance test and (B) percent reduction of glycemic peak at 10 and 15 min. Twenty-four hours fasted rats were submitted to oral glucose tolerance test (A) in the absence (□) or presence (■) of CA or (C) in the presence of glucose solutions with pH 6.2 (□) or 2.8 (▨). Glycaemia was measured at 0 (basal); 5; 10; 15; 30; 45; 60 and 90 min after the oral load of glucose. Each bar represents the mean ± SEM of 8–10 experiments. Differences between control and experimental groups, within fixed time, were analysed by Student's *t*-test for independent samples. $^{*}p < 0.05$ versus control

membranes by the transporter OATP1,[44] depends on passive diffusion to be taken up by hepatocytes. Whether or not the CA molecule does enter these cells, what is certain from our results, taken together, is that in these conditions CA did not attain an intracellular level sufficient to inhibit G-6-Pase.

The current view is that, apart from regulation of the post-fasting glycaemia, another factor important in reducing the risk of developing type 2 diabetes is the regular consumption of low glycemic index food.[24] In this study, CA caused a reduction in the glycemic peak at 10–15 min after ingestion of glucose and this was not due to the lower pH of the glucose solution containing CA. This result can be attributed, at least in part, to an inhibition by CA of intestinal absorption of glucose. In just one other study, using fed type 1 diabetic rats, glycaemia was observed to fall within one to three hours after oral administration of CA.[45] In addition, there is evidence that ingestion of instant coffee, of which CA is a component, reduces the blood glucose during the oral glucose tolerance test in humans[18,22,24,25] and that ingestion of decaffeinated coffee by humans, together with a dose of glucose, tends to lower the glycaemia.[23]

The CA dose used in this study (3.5 mg kg^{-1} bw, in the form of a 10 mM solution) was based on the

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finding that 1 mM CA reduced by 80% the glucose-transport capacity of brush-border membrane vesicles isolated from the small intestine of rats. According to the authors, this phenomenon could be attributed to dissipation of the electrochemical sodium-ion gradient, which provides the driving force for Na^+-dependent glucose transport.[15]

In summary, it has been shown that, whereas ingestion of CA with glucose solution does reduce the glycemic peak, IV injection of a large dose of CA does not affect the blood glucose level and, when CA is infused into the liver, neither is it retained by this organ nor does it affect hepatic production of glucose. These data, taken together, favour the hypothesis that CA may reduce glycaemia and/or the glycemic index of food by attenuating the intestinal absorption of glucose. If this functional property of CA is confirmed in future studies with human subjects, this natural phenolic compound and perhaps coffee itself could be used to reduce the risk of developing type 2 diabetes, by lowering the postprandial hyperglycaemic peak and thus sparing the pancreatic islets from being exhausted by overproduction of insulin.[23–24]

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