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Chicoric acid, a new compound able to enhance insulin release and glucose uptake

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ABSTRACT

Caffeic acid and chlorogenic acid (CGA), a mono-caffeoyl ester, have been described as potential antidiabetic agents. Using *in vitro* studies, we report the effects of a dicaffeoyl ester, chicoric acid (CRA) purified from *Cichorium intybus*, on glucose uptake and insulin secretion. Our results show that CRA and CGA increased glucose uptake in L6 muscular cells, an effect only observed in the presence of stimulating concentrations of insulin. Moreover, we found that both CRA and CGA were able to stimulate insulin secretion from the INS-1E insulin-secreting cell line and rat islets of Langerhans. In the later case, the effect of CRA is only observed in the presence of subnormal glucose levels. Patch clamps studies show that the mechanism of CRA and CGA was different from that of sulfonylureas, as they did not close K_{ATP} channels. Chicoric acid is a new potential antidiabetic agent carrying both insulin sensitizing and insulin-secreting properties.

Type 2 diabetes is a metabolic disorder characterized by abnormal insulin secretion from pancreatic β-cells, associated with insulin resistance of hormone-sensitive tissues. Pharmacological treatments of this disorder are aimed at reducing plasma glucose concentration; it includes insulin secretagogues such as sulfonylureas, glinides and GLP-1 analogues, and insulin-sensitizers which reduce hepatic glucose production (metformin) or enhance glucose utilization by skeletal muscle or adipose tissue (metformin and thiazolidinediones) [1]. No drug currently in use has been shown to intrinsically carry both properties.

The search for new antidiabetic compounds is the focus of intensive investigations, and plants have been discussed as a valuable source of bioactive molecules. Phenolic compounds from plant origin, particularly caffeic and chlorogenic (5, mono-caffeoylquinic) acids are essentially investigated for their antihyperglycemic properties [2–5].

In the present work, we report for the first time the antidiabetic potential of chicoric acid (CRA) a 2,3-dicaffeoyltartaric acid, a phenolic compound extracted from *Cichorium intybus*, also present in several related members of the subfamily Cichonoideae belonging to Asteraceae family [6]. We describe its ability to both increase glucose uptake and enhance insulin secretion in a glucose-dependent manner. The experiments were also conducted with chlorogenic acid (CGA) for comparison purpose.

Materials and methods

Natural compounds and drugs. Chicoric acid were purified from *C. intybus* by Prof. C. Andary (Laboratoire de Botanique et Phytochimie, UMR 5175 of CNRS, Faculté de pharmacie, Montpellier, France). Chlorogenic acid was pursached from Extrasynthèse (Saint Quentin Fallavier, France). Tolbutamide and glibenclamide were purchased from Sigma (Munich, Germany).

L6 myocyte culture and 2-deoxyglucose uptake. In brief, rat myoblast L6 (LGC Promochem, Molsheim, France) were seeded in 12-well plates (10^4 cells per well) in DMEM supplemented with 10% heat-inactivated foetal calf serum (FCS) (Biochrom, Berlin, Germany), 2 mM L-glutamine,100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in a 5% CO_2. At day 3, FCS is decreased to 2% for the differentiation of myocytes during 1 week. As previous describe [7], the day of the experiment, cells were first starved during 4 hours in DMEM supplemented with 0.1% bovine serum albumin

Abbreviations: CRA, chicoric acid; CGA, chlorogenic acid

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(BSA) alone and then incubated during one hour in Krebs-Ringer bicarbonate buffer (KRB), 0.1% BSA, 5 mM glucose supplemented with 100 nM insulin, and the compounds to be tested. Cells were gently washed and then incubated in 0.5 μCi [^{3}H] deoxyglucose in 1 ml KRB per well. Uptake was stopped by three washings in cold PBS and cells were lysed in 0.1 N NaOH. The total protein concentration is evaluated by a Bradford assay [8] and radioactivity is measured. Results are expressed in cpm/mg protein/min.

β-cell culture and measurement of insulin secretion. Rat insulinoma-derived INS-1E cells [9], kindly provided by Prof. C.B. Wollheim (Geneva, Switzerland), were cultured in 24-wells plates (2×10^5 cells by well) in HEPES-buffered RPMI 1640 supplemented with 10% heat-inactivated FCS, 1 mM Na pyruvate, 50 μM β-mercaptoethanol (Sigma, Munich, Germany), 2 mM glutamine, 10 mM HEPES, 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin at 37 °C in a 5% CO_2 during 5–6 days before the experiment (medium replenished on day 3). Prior to the experiment, cells were washed twice and incubated for 90 min in glucose-free KRB, BSA (2%). Next, cells were washed once with glucose-free KRB, BSA (2%) and then incubated for 30 min in KRB, BSA (2%) with glucose at 3.0, 5.8, 11.3 mM or 3 mM plus CRA or CGA at 50 or 100 μg ml^{-1}. Insulin released in the medium and cellular insulin contents from HCl (1.5%)–ethanol (75%) lysa-cells were determined by FRET technology with the Insulin-Kit HTRF® (Cis-Bio International) according to manual procedure. The fluorescence levels were measured on a RUBYstar instrument (BMG LABTECH). Results are expressed in % of insulin cell content.

Rat pancreatic islet experiments. Islets were isolated after collagenase digestion of the pancreas from adult Wistar rats [10]. Immediately after isolation, the islets were pre-incubated for 90 min at 37.5 °C in KRB buffer plus 0.1% BSA and 2.8 mM glucose. Thereafter, batches of three islets were incubated in the presence of the appropriate glucose concentration (2.8 or 8.3 mM) for 60 min in 1 ml medium supplemented or not with CRA or CGA. Insulin released into the supernatants was measured by FRET technology using the Insulin-Kit HTRF®. Results are expressed in ng of insulin/three islets.

Electrophysiology experiments. cRNAs coding mouse Kir6.2 and hamster SUR1 are prepared as described in Hosy et al. [11]. They were co-injected into defoliculated *Xenopus laevis* oocytes, previously isolated in conformity with French regulation (authorization no. 28-03-15 from the "Ministère de l'Agriculture, Direction des Services Vétérinaires"). Two to 6 days after injection, oocytes were manually devitellinized and K_{ATP} channel activity was characterized by the patch clamp technique in the excised inside out configuration [12]. Patch pipettes (about 5 MΩ) contained (in mM) 154 K^+, 146 Cl^-, 5 Mg^{2+} and 10 PIPES pH 7.1. The external solution bathing the cytoplasmic side contained (in mM): 174 K^+, 40 Cl^-, 1 EGTA, 1 Mg^{2+}, 10 PIPES pH 7.1 and 100 methyl sulfonate$^-$ as remaining anions. ATP (potassium salt, Sigma), glibenclamide (Sigma), CRA and CGA were added as specified. Solution perfusion and patch analysis were realized as previously described [11].

Data analysis. Data are expressed as means ± SEM. Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) test at $P < 0.05$, using the Stat Graphics software. Statistics for electrophysiological experiments were conducted using the student's test. P value of <0.05 was considered statistically significant.

Results

CRA and CGA effects on glucose uptake

Data on the effects of CGA and CRA on glucose uptake under insulin-stimulating concentration in L6 cells are reported in Fig. 1. As expected, L6 cells with 100 nM insulin increased basal glucose uptake (+29% in average). In the absence of insulin, CRA and CGA (100 μg ml^{-1}) did not stimulated significantly glucose uptake. In the presence of insulin, CRA and also CGA at 100 μg ml^{-1} induced a significant increase in insulin-induced glucose uptake (+16.4% and +16.1%, respectively, $P < 0.05$).

CRA and CGA effects on insulin secretion

The results of *in vitro* study of the effects of CRA and CGA on insulin released in INS-1 cells are presented in Fig. 2. As expected, elevation of the glucose concentration (from 3 to 5 mM) or addition of tolbutamide (200 μM) to 3 mM glucose stimulated insulin secretion (+74% and +160%, respectively). In the same conditions (3 mM glucose), CRA and CGA (10 and 50 μg ml^{-1}) stimulated insulin secretion to a level close to that obtained with 5 mM glucose.

Insulin secretion from isolated rat pancreatic islets in presence of CRA (panel A) and CGA (panel B) are presented in Fig. 3. Glucose

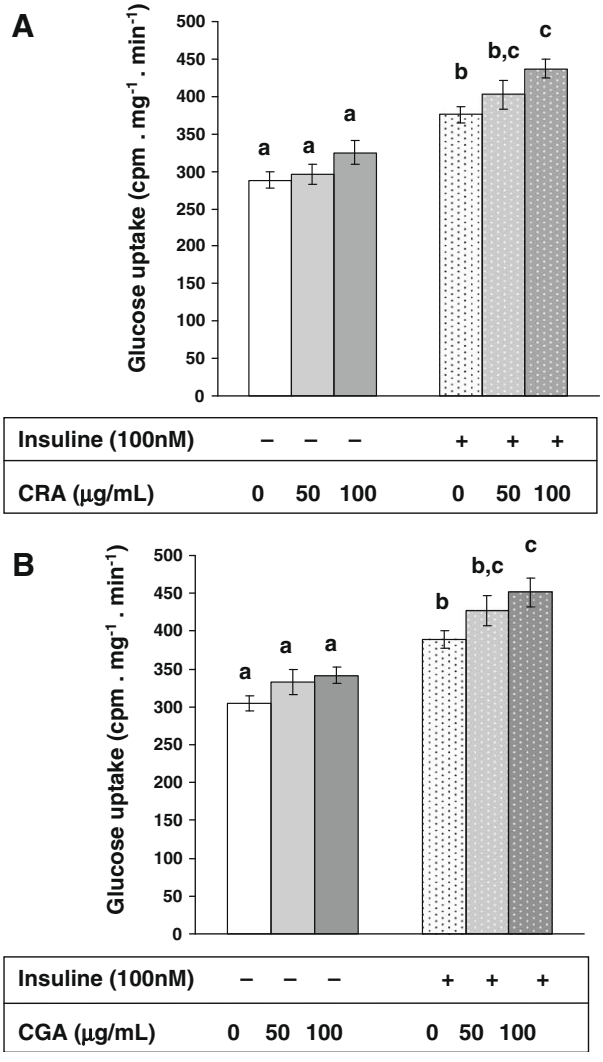
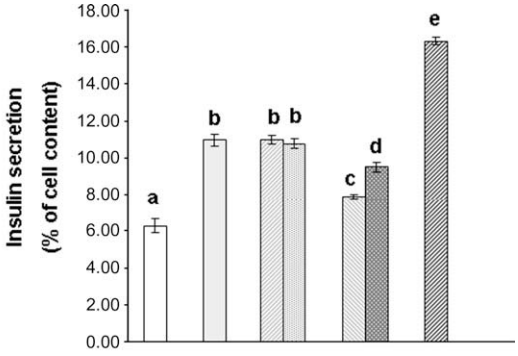


Fig. 1. Effects of CRA and CGA on glucose uptake in L6 myocytes. After 1 week of differentiation, the L6 muscle cells were stimulated during a 1 h treatment with or without 100 nM of insulin in presence of CRA (A) or CGA (B) (50 and 100 μg ml^{-1}). The glucose was then taken up during 5 min and quantified with the [^{3}H] deoxyglucose. Values are means (±SEM) from four separate experiments (each one comprises four independent tests). Bars with different letters (a and b) differ from each other significantly ($P < 0.05$).



Glucose (mM)	3	5	3	3	3
CRA (mg/mL)	-	-	10 50	-	-
CGA (mg/mL)	-	-	-	10 50	-
Tolbutamide (μM)	-	-	-	-	200

Fig. 2. CRA and CGA effects on insulin secretion in INS-1E β-cell line. The cells cultivated in RPMI medium were incubated in glucose-free-RPMI during 2 h. The deprived cells were stimulated with 3 or 5 mM glucose and 3 mM glucose with CRA or CGA (10 or 50 μg ml^{-1}) or Tolbutamide at 200 μM as a positive control. Insulin was quantified by the HTRF-Insulin-Kit (Cis-Bio international). The results are expressed as % of the insulin cell content. Data are means (±SEM) of four independent experiments. Bars with different letters (a, b, and c) differ from each other significantly ($P < 0.05$).

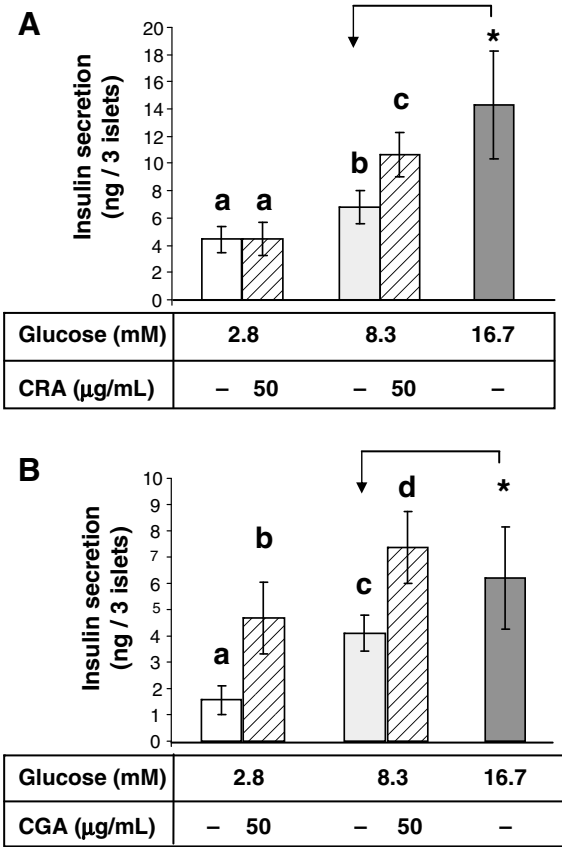


Fig. 3. Effects of CRA and CGA on insulin secretion from isolated rat islets in the presence 2.8 and 8.3 mM glucose. Each test is realized with three islets in Krebs medium with 2.8, 8.3 or 16.7 mM glucose. Effects of CRA or CGA (50 μg ml^{-1}) were tested in 2.8 and 8.3 mM glucose. The results were the means (±SEM) of three independent experiments. Bars with different letters (a, b, and c) differ from each other significantly ($P < 0.05$). *Significantly different value at $P < 0.05$ as compared with 8.3 mM glucose.

elevation level in the medium (2.8 to 16.7 mM) stimulated insulin release. In 8.3 mM glucose, CRA and CGA (50 μg ml^{-1}) increases significantly insulin secretion (1.4- and 1.6-fold increase, respectively). Under 2.8 mM glucose, CGA (50 μg ml^{-1}) stimulated insulin secretion (2.2-fold increase), whereas CRA was ineffective.

CRA and CGA effects on K_{ATP} channels

Xenopus oocytes, injected with both Kir6.2 and SUR1 cRNAs, expressed exogenous potassium ATP-dependent conductance. Fig. 4 illustrates the effects of CRA and CGA in the presence of ATP on the K_{ATP} channel, a well-known target for the sulfonylurea and non-sulfonylurea insulin secretagogues. As expected, the sulfonylurea glibenclamide (100 nM) inhibited potassium conductance in connection with K_{ATP} inhibition. In contrast, CRA and CGA at 10 or 30 μg ml^{-1} (we have previously shown in Fig. 3 that a positive insulin response level is present between 10 and 50 μg ml^{-1} of CRA or CGA) did not significantly modify current properties, even though a slight enhancement of potassium conductance was apparent.

Discussion

Since chlorogenic acid, a mono-caffeoyl ester and caffeic acid, a dicaffeoyl ester were described as antidiabetic compounds, the present study was undertaken to determine the potential of a dicaffeoyl derivative, chicoric acid (CRA), in comparison with chlorogenic acid (CGA). Our data indicate that, similarly to CGA, CRA increases glucose uptake by L6 muscular cells in the presence of insulin; these results are similar to previous report on the insulin-sensitizing effects of caffeic acid and its derivatives on the same model [13]. We also show for the first time that CRA and CGA were able to directly stimulate insulin secretion from pure β-cells (INS-1E cell-line) and from isolated Langerhans islets. The similarity of these properties between CRA and CGA suggests that the effects are not related to their mono- or di-caffeoyl structures. The mechanism of action of caffeoyl derivatives has been recently studied. CGA has been identified as an inhibitor of glucose-6-phosphatase (G6P) in rat liver microsomes [7], a property inducing the inhibition of hepatic glucose production and consequently the reduction of hyperglycemia. The same G6P inhibiting activity has been reported for other CGA derivatives such as S4048 and S3483, which have also been developed for their potential antidiabetic properties [14,15]. G6P inhibition may also induce the increase in glucose transport and utilization, and the stimulation of insulin secretion through an increased production of ATP (see below). Therefore, CRA may possibly act via a similar mechanism. Alternative mechanisms may also include the activation of AMP-activated protein kinase (AMPK), as recently reported for caffeic acid phenethyl ester in muscle cells [13]. However, as shown with metformin [16], activation of AMPK can explain the increased insulin sensitivity, but cannot account for the enhanced insulin secretion [17].

The sulfonylurea insulin secretagogues are widely used in the treatment of type 2 diabetes. Sulfonylureas close the pancreatic β-cell K_{ATP} channels [18,19] through an interaction with the SUR-1 receptor. The subsequent cell membrane depolarization opens voltage-dependent Ca^{2+} channels, increasing cytoplasmic Ca^{2+} and consequent exocytosis of insulin secretory granules. According to this mechanism, insulin secretion can occur even in the absence of hyperglycemia, which potentially leads to hypoglycemia, a frequently occurring side-effect with this class of therapeutic agents [20,21]. In addition, sulfonylureas may decrease cell viability through a SUR1-dependent pathway [22]. The present data indicate that, in contrast to sulfonylureas, CRA and CGA do not close

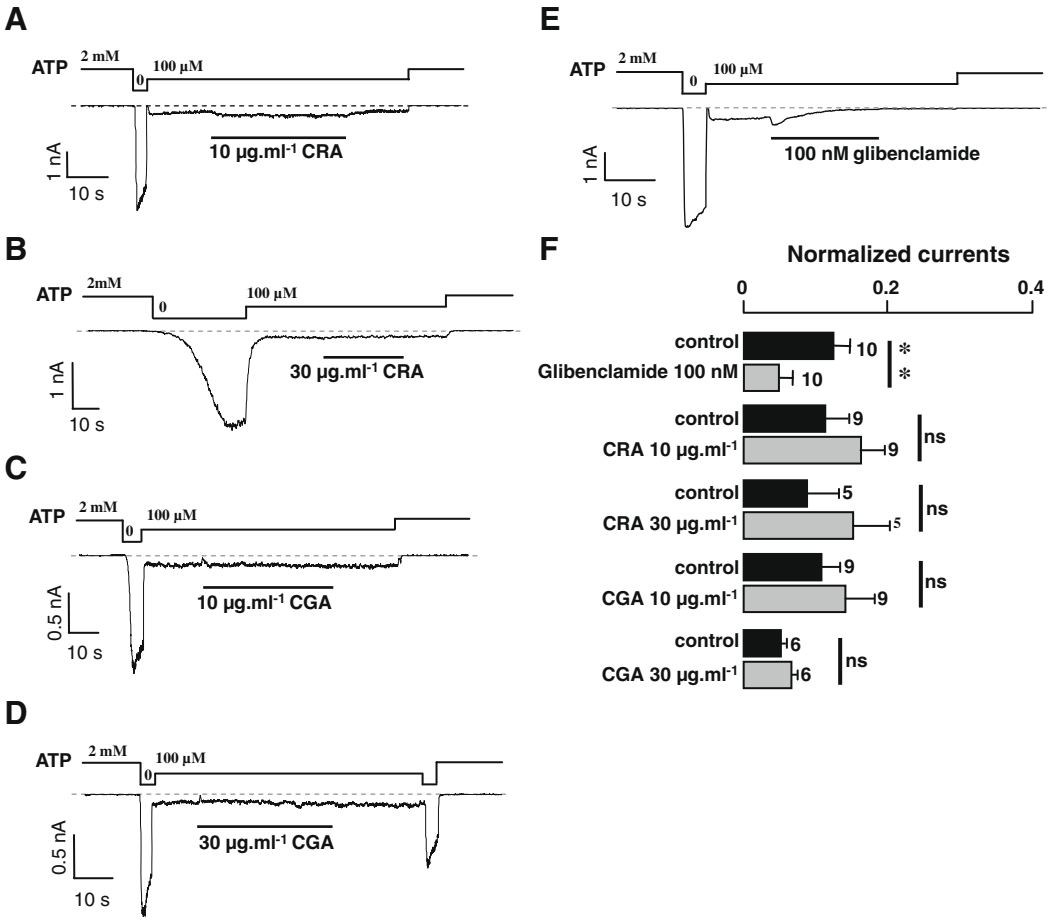


Fig. 4. CRA and CGA effects on the K_{ATP} channels. Representative macroscopic currents recorded from *Xenopus* oocytes membranes (patch clamp technique) co-expressing Kir6.2 and SUR1 subunits in presence of ATP, and 10 and 30 µg ml^{-1} CRA and CGA (A–D). On (E) is shown the inhibitory effect on the K_{ATP} currents of glibenclamide, a well-known antidiabetic drug used as positive control. (F) Average currents recorded in 100 µM ATP in the presence and absence of drugs, normalized to the current measured before in nucleotide-free solution. Numbers upper bars indicate the number of patches included in the averages. Statistical analysis by a student test were realized (*, $P < 0.05$; ns, non-significant).

K_{ATP} channels at stimulating concentrations for insulin secretion, suggesting that a different mechanism of action may be involved. In addition, CRA was totally ineffective on insulin secretion from isolated islets under low glucose concentration (2.8 mM), and its insulinotropic effect clearly appeared at intermediate glucose concentration. Since glucose-6-phosphatase in β-cells is associated with regulation of insulin secretion [22], it may be hypothesized that this effect could be linked to inhibition of glucose-6-phosphatase activity. In addition, glucose-6-phosphatase has been shown to be several folds higher in islets of diabetic animals [23,24].

In summary, the present study shows that the chicoric acid, a dicaffeoyl ester is able to stimulate glucose transport in muscle cells and to increase insulin secretion in a glucose-dependent manner in Langerhans islets. These properties make it a potentially attractive drug candidate for type 2 diabetes, acting on both insulin sensitivity and insulin secretion. Further studies will determine chicoric acid molecular mechanism as well as its effects in normal and diabetic animals.

Disclosures

The results of this study have led to a patent (Tousch D, Ribes G, Andary C). Patent PCT No. WO/2008/022974, August 23, 2000. The patent is held by the Centre National de la Recherche Scientifique of Paris, France.

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