In vitro modulation of pancreatic insulin secretion, extrapancreatic insulin action and peptide glycation by Curcuma longa aqueous extracts

Violet Kasabri¹, Peter R. Flatt², Yasser H. A. Abdel-Wahab²

ABSTRACT

Objective: Medicinal, edible, and aromatic plants have been used as folk remedies in traditional treatments worldwide. This study investigates the antidiabetic efficacy and action mode of Curcuma longa Linn. (Zingiberaceae). Methods: Effects of aqueous extracts (AEs) of C. longa on insulin secretion and action were studied using the insulin-secreting BRIN-BD11 and the adipocyte-like 3T3-L1 cell lines, respectively. In vitro models were employed to evaluate effects on starch digestion using α-amylase/amyloglucosidase and protein glycation. Results: C. longa AEs stimulated basal insulin output and potentiated glucose-stimulated insulin secretion concentration-dependently in the clonal pancreatic beta cell line, BRIN-BD11 (P < 0.001). The insulin secretory activity of plant extract was abolished in the absence of extracellular Ca²⁺ and by inhibitors of cellular Ca²⁺ uptake, diazoxide and verapamil (P < 0.001). Furthermore, the extract increased insulin secretion in depolarized cells and augmented insulin secretion triggered by 3-isobutyl-1-methylxanthine, tolbutamide, and glibenclamide. C. longa AEs lacked insulin mimetic activity but enhanced insulin-stimulated glucose transport in 3T3-L1 adipocytes by 370% (P < 0.001). Similar to aminoguanidine, C. longa AEs (1-50 mg/ml) effected concentration-dependent inhibition of protein glycation (24-70% inhibition, P < 0.001) in vitro. In bioassays of enzymatic starch digestion, C. longa AEs lacked inhibitory effects on α-amylase and α-glucosidase, unlike acarbose, the classical reference drug. Conclusion: This study has revealed that water soluble bioactive principles in C. longa AEs stimulate basal- and potentiate glucose evoked-insulin secretion, enhance insulin action and inhibit insulin glycation, but not starch digestion. Future work assessing the use of C. longa AEs as dietary adjunct or as a source of active antidiabetic agents may provide new opportunities for the combinatorial treatment/prevention of diabetes.

KEY WORDS: Curcuma longa Linn. (Zingiberaceae), insulin action, insulin secretion, peptide glycation, starch digestion

INTRODUCTION

Diabetes mellitus is the most common metabolic disorder affecting millions worldwide. It is recognized as a global major health problem [1]. As alternatives to the available orthodox medicines, plants are considered a potential source for the treatment of diabetes within traditional ethnomedicine practices. Literature surveys summarize the benefit of several ethnobotanicals as anti-diabetic agents in the form of crude extracts and/or isolated pure compounds, which exhibit varying degrees of hypoglycemic or antihyperglycemic bioactivities [2]. Evidently, multiple medicinal herbs, both indigenous or imported, were promoted locally for diabetes traditional medicine. These were closely linked to appreciable prevalence of herbal use among diabetes patients on conventional medicaments in Jordan [3].

A spice originally common in the kitchen and folk medicine for multiple ailments, turmeric, also known as Curcuma longa Linn. (Zingiberaceae), exhibits diverse activities in the clinical trials of lupus nephritis, cancer, diabetes, irritable bowel syndrome, acne, and fibrosis [4-6]. Various phytochemical constituents have been isolated from C. longa, including polyphenols, sesquiterpenes, diterpenes, triterpenoids, sterols, and alkaloids [4,7]. Curcumin is a culinary and medicinal yellowish phenolic spice derived from the rhizome of C. longa, which constitutes 2-5% by weight. It is a natural antioxidant that has diverse pharmacological activities and is perhaps the most-studied component. Although some of the activities of C. longa can be mimicked by curcumin, other activities are curcumin-independent. Importantly, it has been proven that curcumin is a highly pleiotropic molecule, which can be a modulator of intracellular signaling pathways that control cell growth, inflammation and apoptosis [8].
With an excellent safety profile, curcumin has been ascribed anti-inflammatory, anti-microbial, anti-cancer, and neuroprotective anti-Alzheimer efficacies [9]. It is established for its hepatoprotective, nephroprotective [10,11], cardioprotective, hypoglycemic, anti-rheumatic and antidiabetic activities [12,13]. In terms of diabetes, C. longa has been shown to act as antidiabetic in alloxanized rabbits as type 1 diabetes models [14]. In healthy subjects, the ingestion of 6 g C. longa increased postprandial serum insulin levels, but did not seem to affect plasma glucose levels or glycemic index, mainly indicative of C. longa effect on insulin secretion [15]. The turmeric ethanol extract significantly suppressed an increase in blood glucose level in type 2 diabetic KK-A(y) mice and stimulated in vitro human adipocyte differentiation dose-dependently [16].

To further investigate the antidiabetic efficacy and action mode of C. longa, potential effects on insulin secretion and action at the cellular level were evaluated. Furthermore, possible effects on protein glycation and starch digestion were examined in vitro. The results are consistent with the antidiabetic propensities of C. longa being mediated by both pancreatic and extrapancreatic actions.

MATERIALS AND METHODS

Cell Lines, Chemicals and Biochemicals

3T3-L1 fibroblasts were obtained from the American Type Culture Collection (ATCC; VA, USA). Filter paper no.1 (Whatman), vacuum dryer (Savant Speedvac, Savant Instrumentation Incorporation, NY, USA) were used in extract preparations. Wallac 1409 Scintillation Counter was from Wallac (Turku, Finland). Analox GM9 Glucose analyzer was from Analox Instruments (London, UK). Acarbose was obtained from Bayer AG (Wuppertal, Germany). Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant Material

C. longa Linn. (Zingiberaceae) dried plant material was procured from a commercial supplier in Delhi, India, during the winter season, and available from Top-Op Foods Limited (Stanmore, Middlesex, UK). Voucher specimens are kept in Diabetes Research Group, School of Biomedical Sciences, University of Ulster. C. longa plant material was homogenized to a fine powder and stored in opaque screw-top jars at room temperature (20 ± 2°C) until use. For in vitro work, a decoction was prepared by bringing 25 g/l of material to the boil in water. Once boiling, the suspension was removed from the heat and allowed to infuse over 15 min. The suspension was filtered and the volume adjusted so the final concentration was 25 g/l. Sample aliquots of 1 ml of the filtered plant solution were brought to dryness under vacuum. Dried fractions were stored at −20°C until required. Fractions were reconstituted in incubation buffer for subsequent experiments as required.

Insulin Secretion

Insulin release was determined using monolayer of BRIN-BD11 clonal pancreatic cells [17]. BRIN-BD11 cells were grown in RPMI-1640 tissue culture medium containing 11.1 mmol glucose/l, 10% fetal calf serum and antibiotics (50,000 IU penicillin-streptomycin/l), and maintained at 37°C in an atmosphere of 5% CO2 and 95% air. Twenty-four hours prior to acute experiments, cells were harvested and seeded in 24-well plates at a density of 1 × 105 cells/well. Following overnight attachment, culture medium was removed and cells were preincubated for 40 min at 37°C with 1 ml of Krebs ringer bicarbonate (KRB) buffer supplemented with 1.1 mM glucose and 1% bovine serum albumin. Subsequent test incubations were performed for 20 min at 5.6 mM glucose or 16.7 mM glucose or even 0 mM glucose-with or without calcium-using similar buffer supplemented with C. longa aqueous extracts (AEs) and the agents indicated in figures. L-alanine (10 mM) was the reference robust and powerful stimulant of insulin secretion from pancreatic beta cells [17]. Samples were stored at −20°C for subsequent insulin radioimmunoassay [18]. Cell viability was assessed using a modified neutral red assay as described previously [19].

Adipocyte Differentiation and Cellular Glucose Transport

3T3-L1 fibroblasts were used to determine glucose transport [20]. Cells (passages 5-10) were seeded in 12-well plates at a density of 1 × 105 cells/well, maintained at 37 ± 2°C with 5% CO2, and fed every 2 days with Dulbecco's modification of Eagle's medium (DMEM) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and fetal bovine serum (10% v/v). Adipocyte differentiation was initiated as described in details elsewhere by the addition of 1 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.25 μM dexamethasone [19]. Prior to acute tests, cells were incubated in serum free DMEM for 2-3 h to establish basal glucose uptake. Cellular glucose uptake was determined for 15 min at 37°C using KRB buffer supplemented with tritiated 2-deoxyglucose (0.5 μCi/well), 50 mM glucose, insulin, and C. longa AEs as indicated in figures. Hexose uptake was terminated after 5 min by three rapid washes with ice-cold phosphate-buffered saline, after which cells were detached by the addition of 0.1% sodium dodecyl sulfate and subsequently lysed. Scintillation fluid was added to solubilized cell suspensions and mixed thoroughly. Radioactivity was measured on a scintillation counter.

Enzymatic Starch Digestion

To assess in vitro starch digestion, 100 mg of soluble starch was dissolved in 3 ml of distilled water in the absence and presence of C. longa AEs or acarbose 1000 μg/ml as a positive control. Heat stable 0.01% α-amylase from Bacillus leicheniformis (40 μl) was added. After incubation at 80°C for 20 min, the mixture was diluted to 10 ml and 1 ml was incubated with 2 ml of 0.1 M sodium acetate buffer (pH 4.75) and 0.1% amyloglucosidase from Rhizopus mold (30 μl) for 30 min at 60°C. Glucose released was measured on the glucose analyzer.

Kasabri, et al.: In vitro antidiabetic effects of Curcuma longa
Protein Glycation

A simple \textit{in vitro} system was employed to assess protein glycation based on the use of insulin as a model substrate [21]. In brief, 100 \( \mu \)l of human insulin (1 mg/ml) was incubated in 10 mM sodium phosphate buffer (pH 7.4) with 220 mM D-glucose, \textit{C. longa} AEs or aminoguanidine 44 mM (positive control) for 24 h. Sodium cyanoborohydride was added and the reaction was stopped by the addition of 0.5 M acetic acid. Glycated and non-glycated insulins were separated and quantified using reversed-phase high performance liquid chromatography [21].

Statistical Analysis

All results are expressed as mean ± standard error of the mean for a given number of independent observations (\( n \)). Groups of data were compared statistically using unpaired Student’s \( t \)-test. Results were considered as significant if \( P < 0.05 \) and highly significant if \( P < 0.01 \) and \( P < 0.001 \).

RESULTS

Insulin Secretion

Insulin release from BRIN-D11 cells was increased significantly in a dose-dependent manner by \textit{C. longa} AEs over the concentration gradient (5-25 mg/ml) in the presence of 5.6 mM glucose [2.3-4-fold, \( P < 0.001 \), Figure 1]. In this panel of mechanistic sections, \textit{C. longa} AEs were tested at concentrations that were non-toxic as evaluated by modified neutral red assay. However, these concentrations were insulin stimulatory to BRIN-BD11 cells. L-alanine (10 mM) exerted a substantial increase of extracellular Ca\(^{2+}\)-evoked insulin release [\( P < 0.001 \), Figure 2] at 5.6 mM glucose. This effect was abolished by Ca\(^{2+}\) removal [90\% reduction, \( P < 0.001 \), Figure 2]. Similarly, the pronounced insulin stimulatory effects (\( P < 0.001 \)) of \textit{C. longa} AEs (1 and 5 mg/ml) were reduced markedly by 75\% (\( P < 0.001 \)) and 34\% (\( P < 0.01 \)), respectively [Figure 2].

Figure 3 demonstrates that \textit{C. longa} AE (1 mg/ml) alone induced a 3.1-fold (\( P < 0.001 \)) stimulation of insulin release in the absence of glucose. In the presence of 5.6 mM glucose, insulin release was enhanced by 2.2-fold (\( P < 0.001 \)) when \( \beta \)-cells were 20 min exposed to \textit{C. longa} AE. Moreover, it significantly increased 16.7 mM glucose induced-insulin secretion by 3.1-fold [\( P < 0.001 \), Figure 3]. A reduction (52\%) was observed in the insulinotropic action of \textit{C. longa} AE upon exposure to diazoxide, and a decrease of 16\% was exerted by verapamil. The actions of \textit{C. longa} AE were significantly enhanced 2.8-fold (\( P < 0.001 \)) by IBMX [Figure 3]. Insulin release in the presence of 16.7 mM glucose and 30 mM KCl was increased 1.4-fold (\( P < 0.001 \)) by \textit{C. longa} AE. Similarly, \textit{C. longa} AE induced respective 3.3- and 2.7-fold (\( P < 0.001 \)) enhancements in the insulin releasing actions of the oral pharmacotherapeutic hypoglycemic sulfonylureas; tolbutamide and glibenclamide [Figure 3].

Cellular Glucose Transport

In 3T3-L1 adipocytes, Figure 4a shows a sigmoidal concentration dependent response curve of the tritiated deoxyglucose transport activity against the logarithm of \( 10^{-8}-10^{-5} \) M insulin. Insulin (1-5 \( \times 10^{-8} \) M) brought a significant submaximal increase in 2-deoxy-D-[\(^3\)H] glucose (2-DOG) transport. Insulin concentration range of 1-5 \( \times 10^{-6} \) M stimulated the maximal 2-DOG transport [in this cultivated batch of 3T3-L1 adipocytes, Figure 4a]. Insulin (10\(^{-6} \) M) was used in

![Figure 1](image1.png)

**Figure 1:** Modulatory effects of \textit{Curcuma longa} aqueous extracts (0.01-25 mg/ml) on pancreatic \( \beta \)-cells BRIN-BD11 insulin release \textit{in vitro}. Such augmentations of glucose stimulated insulin secretion following acute herbal treatments were evaluated by insulin radioimmunossay. Each bar indicates the mean ± standard error of the mean of eight independent observations. ***\( P < 0.001 \) compared to 5.6 mM glucose alone (basal control)
the subsequent investigations of the effects of C. longa AE on peripheral hexose transport, to ensure a broad activity window between the maximal ($10^{-4}$ M) insulin and the far submaximal ($1 \times 10^{-9}$ M) insulin concentrations. As shown in Figure 4b, insulin ($10^{-9}$ M) induced a 1.3-fold increase in the basal 2-DOG transport, compared to basal incubations. C. longa AE effected a 1.3-fold increase in basal 2-DOG transport, compared to untreated basal (no insulin) incubations, not significantly different from $10^{-9}$ M insulin-stimulated 2-DOG transport [Figure 4b]. Yet in the 20 min co-treatment with $10^{-6}$ insulin, C. longa AE increased the insulin-evoked glucose transport by 3.7-fold ($P < 0.001$).

Enzymatic Starch Digestion and Protein Glycation

Using acarbose (1 mg/ml) as a positive control, glucose liberation from starch was abrogated by 98.9% (1.1 ± 0.5% glucose liberated compared with 99.6 ± 1.6% for control, $P < 0.001$). Figure 5 confirms the lack of C. longa AE’s effects on starch digestion hydrolases, namely α-amylase and α-glucosidase. The classical antiglycation drug, aminoguanidine (44 mM), inhibited insulin glycation substantially by 81% ($P < 0.001$). More interestingly, C. longa AE exerted a significant dose-dependent inhibitory effect on insulin glycation ($P < 0.001$; Figure 6). Insulin glycation % inhibition increased markedly from 24% ($P < 0.001$) with 1 mg/ml to 70% ($P < 0.001$) with 50 mg/ml C. longa AE.

DISCUSSION

In streptozotocin (STZ)-diabetic rats, freeze dried rhizome powder of C. longa in milk was proven hypoglycemic and hypolipidemic in mouse pancreas and muscle tissues, respectively [22]. C. longa AE was shown to exhibit both insulin releasing and insulin-like actions [23]. Curcumin was also proven for in vitro insulinogenic efficacy in STZ-treated rat pancreatic islets [24]. Thus, curcumin and C. longa administered to alloxan diabetic rats could reduce the blood sugar substantially [25]. In this study, acute incubations with glucose responsive BRIN-BD11 cells to investigate the possible pancreatic effects of C. longa AE on β-cell function in vitro, revealed a dose dependent Ca^{2+}-evoked stimulation of insulin secretion. Although this action was not glucose dependent, abolition of secretion by chelation of extracellular Ca^{2+} or the absence of adverse effects on cell viability argue against a simple cytotoxic action of C. longa AE at the concentrations employed.
Kasabri, et al.: In vitro antidiabetic effects of Curcuma longa

In vitro antidiabetic effects of Curcuma longa

Studies to evaluate the possible mechanisms underlying the insulin releasing actions of C. longa AEs indicated a broad similarity to the effects of the sulfonylurea drugs. The inhibitory effects of diazoxide on β-cells arising from activation K<sub>ATP</sub> channels indicate involvement of K<sub>ATP</sub> channel closure in the stimulatory actions of C. longa AEs [26]. Interestingly, the acute stimulatory effects of C. longa AEs were also evident at 16.7 mM glucose in β-cells depolarized by 30 mM KCl, indicating K<sub>ATP</sub> channel dependent effects as noted also for sulfonylureas [27]. The observations that verapamil decreased the ability of herbal extract to stimulate insulin output further support the hypothesis that the insulinotropic actions are associated with modulation of β-cell Ca<sup>2+</sup> handling via voltage dependent Ca<sup>2+</sup> channels. C. longa AEs also amplified the late stages of insulin release in the presence of IBMX suggesting that cyclic adenosine monophosphate synergistically complements C. longa-evoked insulin secretion. The fact that the insulin releasing effects of C. longa AEs are physiological suggests that the extract contains water soluble compounds that could be purified for the use in the treatment of type 2 diabetes.

Insulin resistance is considered to be a key pathogenic factor in type 2 diabetes and an obvious target for antidiabetic medication [28]. Insulin resistance also leads to other disorders such as obesity, dyslipidemia, hypertension and cardiovascular disease, collectively termed as insulin resistance associated disorder [29]. Although thiazolidinediones (TZDs) showed significant therapeutic insulin sensitizing potential, their use has already been restricted due to several undesirable side-effects such as hepatotoxicity cardiomegaly and hemotoxicity [30]. Metformin is the only globally available drug currently used for improving insulin action clinically [31]. Interestingly, cinnamon extracts
have been shown also to improve insulin receptor function by activating insulin receptor kinase and inhibiting insulin receptor phosphatase, leading to increased insulin sensitivity [32]. This further illustrates the potential of plant constituents to augment insulin action. In this study, C. longa AEIs significantly enhanced the acute glucose transport in rat adipocytes in a magnitude similar to or greater than $10^{-8} \text{ M}$ insulin. This indicates that C. longa AEIs contain molecules with significant insulin like efficacies. As with insulin, an induction time of 15 min was needed for the stimulatory effect of C. longa AEIs on basal glucose transport to become apparent. This suggests that C. longa AEIs may promote cellular glucose transport by a mechanism similar to that of insulin. However, this pathway is apparently very different from that used by other antidiabetic drugs, such as TZDs, which are insulin dependent and enhance insulin stimulated glucose transport by activating peroxisome proliferator-activated receptor gamma, thereby up-regulating glucose transporter type 4 gene expression. Comparing C. longa AEIs to insulin, the responses observed using plant/insulin combined treatments were greater than additive, increasing sensitivity to insulin by 3.7-fold. This indicates synergism due to insulin sensitization properties of C. longa AEIs. Previously, C. longa AEIs have been reported to induce step-wise stimulation of glucose uptake from mouse abdominal muscle tissues in the presence and absence of insulin [23]. In comparison to our acute incubations with nanomolar insulin concentrations, a 1000-fold greater insulin dose was needed in insulin sensitizing studies of C. longa hydroalcoholic extracts [33]. Taken together, these data suggest that water soluble components of C. longa AEIs may be useful for alleviation of insulin resistance and in the study of the pathways enhancing to glucose utilization [34].

When considering synergistic antioxidative-antiglycation properties of C. longa, it is interesting to note that curcumin/curcinomids in turmeric have proven to be strong antioxidants and inhibitors of advanced glycation end products [35]. In fact, C. longa was preventive of carbon tetrachloride generated-oxidative stress in rats via elevating antioxidative potential and decreasing lipid peroxidation [36]. In addition, dose-dependent anti-apoptotic effects were ascribed to the protective effects of C. longa against STZ-induced oxidative damage in pancreatic insulin-producing RINm5F cell line [37]. Turmeric and curcumin supplementation significantly reduced the oxidative stress and glycosylated hemoglobin levels encountered by the alloxan-diabetic rats [25]. Thus the prominent anti-insulin glycation propensities of C. longa observed in the current study are probably due to its reported strong antioxidative potencies.

In the previous studies, C. longa rhizome water extract was reported to have concentration dependent inhibitory effect on activity of human pancreatic α-amylase with an half maximal inhibitory concentration (IC_{50}) value of 0.16 $\mu$g/ml [38]. Bisdemethoxycurcumin of C. longa was suggested to act as a small molecule inhibitor of porcine and human pancreatic α-amylase with IC_{50} values of 0.026 and 0.025 mM, respectively [39]. Potent α-glucosidase inhibition has also been reported for natural curcinomids and synthetic curcumin analogs of C. longa [40,41]. In addition, Ar-Turmerone, the major volatile component in the rhizome, and turmerin, the rhizome water soluble peptide, showed potent α-glucosidase inhibition [42,43]. In the present study, acarbose was confirmed as a potent inhibitor of in vitro starch digestion [19,44]. Surprisingly, C. longa AEIs were inactive in our bioassay system of enzymatic starch hydrolysis using successive thermophilic α-amylase and α-glucosidase incubations.

This study has highlighted that the C. longa AEIs stimulate basal insulin release and potentiate glucose-evoked Ca^{2+} regulated insulin secretion. C. longa AEIs combined with $10^{-6}$ M insulin potentiated insulin action in 3T3-L1 adipocytes. Extracts of C. longa also exerted a significant dose-related inhibition of protein glycation. Future work is required to purify and characterize the active water soluble components of C. longa responsible for these actions in order to bring forward potential novel agents for integrated diabetes management.

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