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Curcumin stimulates glucagon-like peptide-1 secretion in GLUTag cells via Ca^{2+}/calmodulin-dependent kinase II activation

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is a hormone secreted from enteroendocrine L-cells. Enhancing GLP-1 action is an important target for prevention and treatment of type 2 diabetes. Several approaches (GLP-1 analogs, dipeptidyl peptidase IV inhibitors) are being used to develop therapeutic agents using GLP-1 action for the treatment of diabetes. However, an alternative approach is to increase endogenous GLP-1 secretion through modulation of the secretory mechanism in intestinal L cells by pharmaceutical agents or dietary ingredients. In the present study, we demonstrate that curcumin, a yellow pigment isolated from the rhizomes of *Curcuma longa* L, significantly increases GLP-1 secretion in GLUTag cells, and we clarified the structure–activity relationship using curcumin derivatives. Also, concerning the secretory mechanism, the significant increase in GLP-1 secretion by curcumin involved the Ca^{2+}–Ca^{2+}/calmodulin-dependent kinase II pathway, and was independent of extracellular signal-regulated kinase, PKC, and the cAMP/PKA-related pathway. These findings provide a molecular mechanism for GLP-1 secretion mediated by foods or drugs, and demonstrate a novel biological function of curcumin in regards to GLP-1 secretion.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a gut hormone secreted from enteroendocrine L-cells, and is recognized as one of the incretins that stimulate glucose-dependent insulin secretion and β-cells proliferation [1,2]. The role of GLP-1 in the metabolic response, including glucose homeostasis, has been established as potentially important in type 2 diabetes patients. Therefore, enhancing GLP-1 action is useful for the prevention and treatment of type 2 diabetes. Several approaches are being used to develop therapeutic agents using GLP-1 action. GLP-1 analogs have been demonstrated to improve glycemic control in type 2 diabetes patients [3,4]. However, GLP-1 analogs are not orally bioavailable. Circulating GLP-1 has a very short half-life due to inactivation by the enzyme dipeptidyl peptidase IV (DPP-4) [5]. DPP-4 is an enzyme responsible for N-terminal cleavage of intact GLP-1 [6]. Several DPP-4 inhibitors have been developed to extend the half-life of endogenously secreted GLP-1 in the blood stream, resulting in ameliorating hyperglycemia in type 2 diabetes patients [7].

These approaches are effective in controlling blood glucose levels in type 2 diabetes patients. However, an alternative approach can be to increase endogenous GLP-1 secretion through modulation of the secretory mechanism in intestinal L cells by pharmaceutical agents or dietary factors. This strategy may provide a novel therapeutic opportunity, decrease the doses of other diabetic medicines, and help prevent diabetes. Several nutrients or small molecules increase GLP-1 secretion. Some fatty acids stimulate GLP-1 secretion *in vitro* and *in vivo* [8–10]. It is well known that glutamine is a potent GLP-1 secretagogue [11,12]. Protein hydrolysates have also been reported to induce GLP-1 secretion [13–16]. PPARβ/δ synthetic agonists (GW501516 or GW0742) induce GLP-1 release in intestinal L cells via increasing proglucagon expression [17]. Although there are several studies showing increased GLP-1 secretion by nutrients or drug candidates, there has been little evidence that non-nutritive food compounds, not nutrients themselves, directly increase GLP-1 secretion. Also, the underlying mechanism of GLP-1 secretion is unclear and controversial.

Curcumin is a yellow pigment isolated from the rhizomes of *Curcuma longa* L. There have been many studies on the biological functions of curcumin [18]. Curcumin intervention in a prediabetic population significantly lowered the number of prediabetic

Abbreviations: 2-APB, 2-aminoethyl diphenylborinate; BMC, bisdemethoxycurcumin; CaMKII, Ca^{2+}/calmodulin-dependent kinaseII; DMC, demethoxycurcumin; DPP-4, dipeptidyl peptidase IV; ERK, extracellular signal-regulated kinase; Fos, forskolin; GLP-1, glucagon-like peptide-1; IBMX, 3-isobutyl-1-methylxanthine; IP_3, inositol 1,4,5-trisphosphate; KRB, Krebs–Ringer bicarbonate buffer; KRH, Krebs–Ringer phosphate-HEPES buffer; MEK, mitogen activated or extracellular signal-regulated protein kinase; THC, tetrahydrocurcumin.

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individuals and appeared to improve overall function of β-cells [19]. However, the molecular mechanisms underlying this effect remain unclear. Taking this information into account, we hypothesized that curcumin has multiple biological functions and may aid in GLP-1 secretion and significantly contribute to the prevention and treatment of diabetes.

In the present study, we demonstrated that curcumin significantly increases GLP-1 secretion in GLUTag cells and clarified the structureactivity relationship using curcumin derivatives. Moreover, this increase involves the Ca^{2+}/calmodulin-dependent kinase II (CaMKII) pathway, and is independent of the extracellular signal-regulated kinase (ERK), PKC, PKA, and cAMP-related pathways.

2. Materials and methods

2.1. Chemicals

The purity of all administered chemicals was over 98%. Curcumin, forskolin (Fos), ionomycin, 3-isobutyl-1-methylxanthine (IBMX), sodium dantrolene, verapamil chloride, H-89, KN-93, and Gö6983 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Demethoxycurcumin (DMC) and bisdemethoxycurcumin (BMC) were purchased from Nagara Science (Gifu, Japan). Tetrahydrocurcumin (THC) was obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). The chemical structures of these curcuminoids are shown in Fig. 1A. 2-Aminoethyl diphenylborinate (2-APB) was purchased from Cayman Chemical (Ann Arbor, MI). Fluo-4AM was obtained from Dojindo (Kumamoto, Japan). Anti-phospho-CaMKII (Thr286), anti-CaMKII, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture and GLP-1 secretion

The murine GLUTag L cell line (a gift from Dr. D.J. Drucker, University of Toronto, Toronto, Canada) was cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. For secretion experiments, 80% confluent cells were starved with Krebs–Ringer bicarbonate buffer (KRB; 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, 22 mM $NaHCO_3$) with 0.5% (w/v) fatty acid free BSA for 1 h. Then the cells were incubated with various test compounds in KRB containing 0.5% of fatty acid free BSA for 2 h [10]. After treatment, medium was collected and centrifuged at 800*g* for 5 min at 4 °C to remove any floating cells. Secreted total GLP-1 was assayed using an ELISA specific for GLP-1(7–36 amide) and GLP-1(7–37) assay kit (GLP-1 ELISA-kit, Millipore, St. Charles, MS) according to the manufacturer's instructions.

2.3. Measurement of intracellular Ca^{2+} levels

GLUTag cells were detached from plates with a trypsin/EDTA mix and washed with fresh cell culture medium by centrifugation at 800×*g* for 5 min. Cells were then resuspended in Krebs–Ringer-HEPES (KRH) buffer (50 mM HEPES pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1 mM glucose) containing 2 μM of Fluo-4AM, and the cells were incubated for 60 min at 37 °C in the dark. Cells were washed and resuspended in KRH buffer at concentration of 2×10^6 cell/mL and transferred to a cuvette for whole-cell population calcium measurement. The Fluo-4 fluorescence was recorded on spectrofluorophotometer (RF-5300PC, Shimadzu, Kyoto, Japan) with excitation at 495 nm and emission at 518 nm.

2.4. Treatment of GLUTag cells and immunoblot analysis of various proteins

GLUTag cells were placed in serum-free DMEM containing 1% BSA for 3 h. After incubation, the cells were treated with vehicle (0.1% DMSO) or curcumin for the indicated time periods and conditions. After treatment, the cells were lysed [20]. Supernatant aliquots were treated with Laemmli sample buffer for 5 min at 100 °C [21]. The samples (20 μg protein) were then loaded into a SDS–PAGE system. The resulting gels were transblotted onto

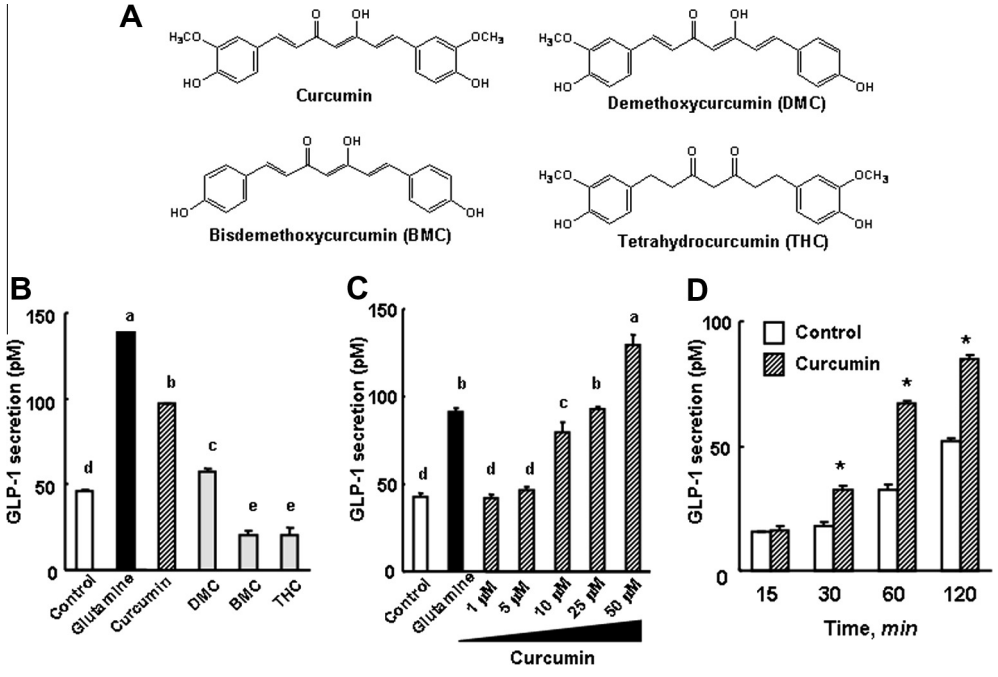

Fig. 1. (A) Chemical structure of curcumin and its derivatives. (B–D) Secreted GLP-1 concentration in media in GLUTag cells. (B), Curcumin and various derivatives (25 μM), positive control (10 mM glutamine), or vehicle (0.1% DMSO) were administered for 2 h. After that, GLP-1 concentration in media was determined using ELISA. (C) The effect of varying concentrations of curcumin on GLP-1 secretion. (D) The time effect of administered curcumin (25 μM) or vehicle (0.1% DMSO) on GLP-1 secretion in media. Values are means ± SEM, $n = 3$. Values without a common letter are significantly different at $P < 0.05$ (B, C). *Significantly different from control at the indicated period, $P < 0.05$ (D).

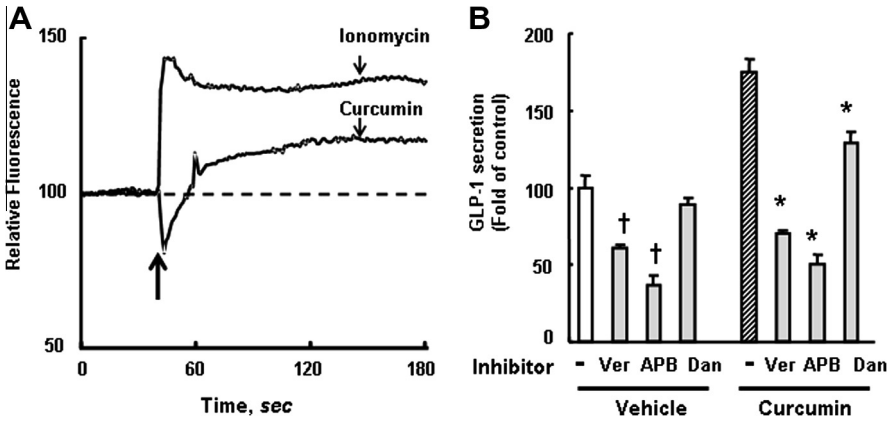

Fig. 2. (A) Intracellular Ca^{2+} response of GLUTag cells treated with curcumin. GLUTag cells were loaded with Fluo4-AM and fluorescence was analyzed after treatment with curcumin (25 μM) or ionomycin (4.2 μM). (B) The effect of various Ca^{2+} signaling inhibitors (verapamil, 20 μM; 2-APB, 50 μM; and dantrolene, 25 μM) on curcumin-stimulated GLP-1 secretion in GLUTag cells. GLUTag cells were pre-treated with vehicle (0.1% DMSO) or inhibitors for 15 min, followed by treatment with vehicle or curcumin (25 μM) for 2 h. The GLP-1 levels in media were determined using ELISA. Secreted GLP-1 levels in media are expressed as the fold of the control (=1.0). Values are means ± SEM, n = 3–6. *Significantly different from curcumin alone ($P < 0.05$), †significantly different from control (vehicle) ($P < 0.05$).

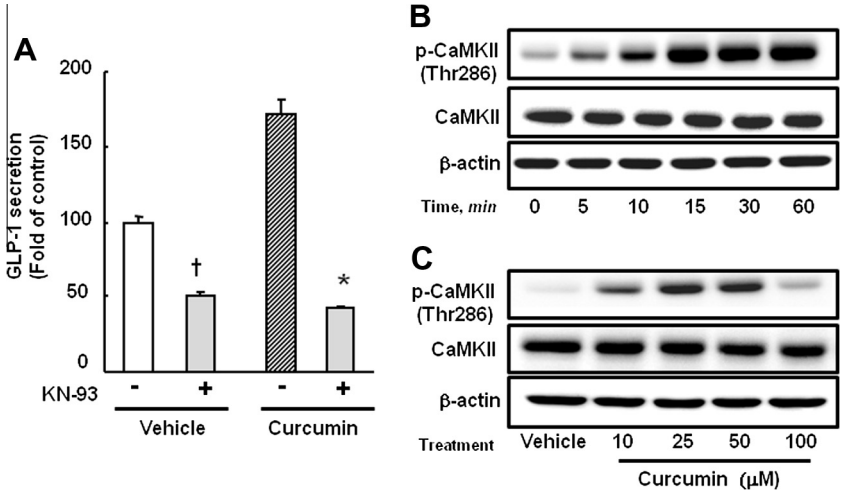

Fig. 3. (A) The effect of CaMKII inhibitor (KN-93) on curcumin-stimulated GLP-1 secretion in GLUTag cells. GLUTag cells were pre-treated with vehicle (0.1% DMSO) or KN-93 (10 μM) for 15 min, followed by treatment with vehicle or curcumin (25 μM) for 2 h. The GLP-1 levels in media were determined using ELISA. Secreted GLP-1 levels in media are expressed as the fold of the control (=1.0). Values are means ± SEM, n = 3–6. *Significantly different from curcumin alone ($P < 0.05$), †significantly different from control (vehicle) ($P < 0.05$). (B, C) Immunoblot analysis of the effect of curcumin duration (B) and dose (C) on phosphorylated CaMKII, total CaMKII, and β-actin protein. The cells were treated with vehicle (0.1% DMSO), or 25 μM of curcumin for various durations (B) and with varying concentrations of curcumin for 15 min (C).

nitrocellulose membranes and the sheets were probed with various antibodies for 16 h at 4 °C. They were then reacted with horseradish peroxidase-conjugated anti-rabbit antibody, the immunoreactivity was visualized using ECL reagent (GE Healthcare Bioscience, Tokyo, Japan), and the relative density was evaluated with Multi Gauge Ver 3.0 Densitograph Software (Fuji Film, Tokyo, Japan). The experiments were performed in triplicate, and representative results are shown.

2.5. Measurement of intracellular cAMP levels

Treated cells were extracted by 0.1 M HCl to avoid degradation of cAMP, and cAMP levels of the extract were measured using a cAMP assay kit (Enzo Life Sciences, Plymouth Meeting, PA) according to the manufacturer's instructions.

2.6. Statistical analysis

The data was expressed as means ± SEM. Differences among means were analyzed by the Tukey–Kramer test. In the case of Fig. 1D, the differences among the means were analyzed by Student's t-test. For all tests, differences with P values <0.05 were considered significant.

3. Results

3.1. Curcumin stimulates GLP-1 secretion from GLUTag cells

First, we examined whether various curcuminoids (curcumin, DMC, BMC, and THC) stimulated GLP-1 secretion. Treatment of GLUTag cells with various curcuminoids showed that curcumin administration resulted in a significant increase in the secretion of GLP-1 (Fig. 1B). DMC, which is the demethoxylated form, also significantly stimulated GLP-1 secretion; however, the secretion level was significantly decreased compared with the curcumin-stimulated secretion level. BMC, which has no methoxyl moiety, and THC, which has two methoxyl moieties, but does not have a β-diketone structure, did not stimulate GLP-1 secretion. Curcumin stimulated GLP-1 secretion in a concentration-dependent manner (Fig. 1C). Analysis of the time effect showed that GLP-1 secretion was significantly increased by the administration of curcumin after 30 min compared to the control (Fig. 1D). The treatment of GLUTag

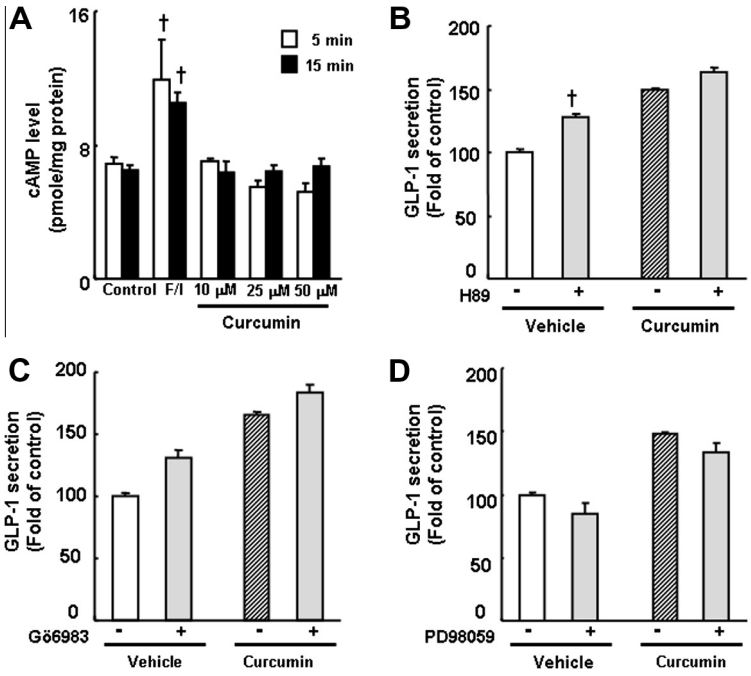

Fig. 4. (A) Cytosolic cAMP concentration of GLUTag cells treated with vehicle (0.1% DMSO), Fos (10 μM) + IBMX (10 μM) (F/I), or curcumin (25 μM) after 5 or 15 min. (B–D) The effect of PKA (H89, 10 μM), PKC (Gö6983, 1 μM), and MEK (PD98059, 25 μM) inhibitors on curcumin-stimulated GLP-1 secretion in GLUTag cells. GLUTag cells were pre-treated with vehicle (0.1% DMSO) or inhibitors for 15 min, followed by treatment with vehicle or curcumin (25 μM) for 2 h. The GLP-1 levels in media were determined using ELISA. Secreted GLP-1 levels in media are expressed as the fold of the control (=1.0). Values are means ± SEM, n = 3–6. †Significantly different from control (vehicle) ($P < 0.05$).

cells with up to 50 μM of each test compound for 2 h produced no cytotoxicity (cell viability was greater than 98%) according to an auto live cell counter system (Countess, Life Technologies, Tokyo, Japan) (data not shown).

3.2. Intracellular Ca^{2+} response of GLUTag cells to curcumin

GLP-1 secretion is regulated by various signaling pathways. Exocytosis, including GLP-1 secretion, is provided by Ca^{2+}, and increases in the cytosolic Ca^{2+} level are achieved by mobilization of intracellular Ca^{2+} stores or extracellular influx into cells via Ca^{2+} channels [22]. To investigate whether curcumin affected intracellular Ca^{2+} levels, cells were loaded by incubation with Fluo4-AM, and the fluorescence was followed during the application of curcumin. The administration of curcumin (25 μM) increased the fluorescence compared to the basal level (Fig. 2A).

3.3. Curcumin-induced GLP-1 secretion is reduced by Ca^{2+} signaling inhibitors

To examine the GLP-1 secretion-related Ca^{2+} response triggered by curcumin, we investigated the effect of various Ca^{2+} signaling inhibitors on curcumin-stimulated GLP-1 secretion. Pre-treatment with verapamil (an L-type Ca^{2+} channel blocker), 2-APB (a inositol 1,4,5-trisphosphate (IP_3) receptor antagonist), or dantrolene (a ryanodine receptor (RyR) antagonist) to inhibit Ca^{2+} release from intracellular Ca^{2+} stores significantly reduced curcumin-stimulated GLP-1 secretion (Fig. 2B).

3.4. Phosphorylation of CaMKII is involved in curcumin-stimulated GLP-1 secretion

It is well known that Ca^{2+} is an important regulator of exocytosis [23]. One of the common targets of Ca^{2+}-mediated signaling is CaMKII, which is regulated via binding of Ca^{2+}-calmodulin resulting in autophosphorylation of stimulatory and inhibitory sites [24]. Recent studies have shown that activation of CaMKII is involved in drug candidate-stimulated insulin secretion in β-cells [25,26], and the mechanism of curcumin-stimulated GLP-1 secretion may be similar to that of insulin secretion from β-cells. Therefore, we examined the effect of a CaMKII inhibitor on curcumin-induced GLP-1 secretion. Pre-treatment of GLUTag cells with a CaMKII inhibitor (KN-93) completely blocked curcumin-induced GLP-1 secretion (Fig. 3A). As curcumin-stimulated GLP-1 secretion was abolished by pre-treatment with the CaMKII inhibitor, we next examined the effect of curcumin on phosphorylation of CaMKII. Treatment of GLUTag cells with curcumin significantly induced phosphorylation of CaMKII protein in a time-dependent manner; phosphorylation began at 5 min and reached nearly maximal levels by 15 min. The phosphorylation was maintained during the experimental period (Fig. 3B). The administration of curcumin also induced phosphorylation of CaMKII protein in a dose-dependent manner; however, it decreased at 100 μM because of cytotoxicity (Fig. 3C). This response is consistent with that of curcumin-stimulated GLP-1 secretion into the media.

3.5. Intracellular levels of cAMP, PKA, PKC, and ERK are not involved in curcumin-stimulated GLP-1 secretion

Previous studies have shown that activation of PKA and PKC pathways in enteroendocrine cells stimulates GLP-1 secretion [27,28]. Also, G protein-coupled receptors (GPCR), such as GPCR40, 119, or 120, are involved in the stimulation of GLP-1 accompanying an increase in intracellular cAMP levels [26–29]. Furthermore, recent studies have shown that GLP-1 secretagogues are stimulated by the MEK-ERK pathway [14,33,34]. To test other possible mechanisms for curcumin-induced GLP-1 secretion, we examined the involvement of these pathways. First, we examined the effect of curcumin on intracellular cAMP levels. Treatment of GLUTag cells with a positive control (Fos + IBMX) resulted in significant elevation of intracellular cAMP levels at 5 and 15 min; however, treatment with curcumin did not affect intracellular cAMP levels in

GLUTag cells (Fig. 4A). Also, curcumin-induced GLP-1 secretion was not significantly affected by pre-treatment with a PKA inhibitor (H-89), a PKC inhibitor (Gö6983), or a MEK inhibitor (PD98059) (Fig. 4B–D).

4. Discussion

The role of GLP-1 in the metabolic response, including glucose homeostasis, has been established, and enhancing GLP-1 action is a potential means for the prevention and treatment of type 2 diabetes. Several approaches (GLP-1 analogs and DPP-4 inhibitors) are being used as therapeutic modalities using GLP-1 action. These approaches have great potential to control blood glucose levels in type 2 diabetic patients. Another approach is to increase endogenous GLP-1 secretion through the modulation of the secretory mechanism in intestinal L cells by pharmaceutical agents. However, there has been little evidence that non-nutritive food ingredients themselves can directly increase GLP-1 secretion. Also, the underlying mechanism of GLP-1 secretion has been unclear and controversial. The present study demonstrated that curcumin has a significant effect on GLP-1 secretion and acts in a unique pharmacological manner, conferring the ability to regulate glucose homeostasis.

The administration of curcumin significantly stimulated GLP-1 secretion in GLUTag cells. Our findings on the structure–activity relationship of curcumin derivatives clearly indicate that at least one methoxyl moiety at the aromatic ring and the β-diketone structure are essential for significant stimulation of GLP-1 secretion. This is the first study to show that curcumin can enhance GLP-1 secretion, demonstrating an additional possible mechanism for an anti-diabetes effect via GLP-1.

These findings raised the question of how curcumin might increase GLP-1 secretion in GLUTag cells. Two possible molecular mechanisms for the curcumin-stimulated GLP-1 secretion are: (1) elevation of cytosolic Ca^{2+} via endogenous Ca^{2+} store mobilization and/or extracellular influx from L-type Ca^{2+} channels, resulting in activation of CaMKII which may be involved in GLP-1 secretion as well as insulin secretion; and (2) stimulation of the cAMP/PKA, PKCζ, or ERK pathways. In this study, we demonstrated that curcumin stimulated the elevation of intracellular Ca^{2+} levels and that curcumin-induced GLP-1 secretion was significantly inhibited by the pre-administration of an IP_3 receptor antagonist (2-APB), a RyR antagonist (dantrolene), and an L-type Ca^{2+} channel blocker (verapamil). One important target of Ca^{2+}-mediated signaling molecules is CaMKII, and CaMKII is regulated via binding to Ca^{2+}-calmodulin resulting in autophosphorylation of stimulatory and inhibitory sites [24]. Interestingly, there are some reports showing that activation of CaMKII mediated by cytosolic Ca^{2+} elevation is involved in stimulation of insulin secretion in a β-cell line [25,26], suggesting that GLP-1 secretion might result from CaMKII activation via cytosolic Ca^{2+} elevation, similar to insulin secretion. Significant GLP-1 secretion induced by curcumin was abolished by the pre-administration of a CaMKII inhibitor (KN-93). Furthermore, treatment of GLUTag cells with curcumin induced phosphorylation of CaMKII in a time- and concentration-dependent manner. These results indicate that curcumin-induced GLP-1 secretion is regulated by the Ca^{2+}-CaMKII pathway in GLUTag cells.

It is also possible that curcumin-stimulated GLP-1 secretion is regulated by the cAMP/PKA, PKCζ, or MEK-ERK pathways. However, treatment of GLUTag cells with curcumin did not affect intracellular cAMP levels, and also pre-treatment with specific inhibitors of PKA, PKC, and MEK (H-89, Gö6983, and PD98059, respectively) did not significantly affect curcumin-stimulated GLP-1 secretion. These results indicate that curcumin-stimulated GLP-1 secretion is not regulated by the cAMP/PKA, PKCζ, or MEK-ERK pathways.

The present study raises another question regarding the primary molecular target of curcumin. Also, these findings lead us to ask whether curcumin can significantly stimulate GLP-1 secretion *in vivo*. Several studies have shown that GPCR agonists stimulate GLP-1 secretion in intestinal L cells [29–32]. Curcumin may activate GPCR and stimulate GLP-1 secretion via the Ca^{2+}-CaMKII pathway. As another possible mechanism, lipid soluble curcumin can be easily incorporated into cells, and incorporated curcumin may directly modulate this pathway and result in the stimulation of GLP-1 secretion.

In conclusion, we demonstrated that curcumin significantly stimulates GLP-1 secretion in GLUTag cells, and this significant increase involves the Ca^{2+}-CaMKII pathway and is independent of the cAMP/PKA, PKC, and MEK-ERK pathways. These findings provide a possible molecular mechanism of GLP-1 secretion mediated by foods or drugs, and demonstrate a novel biological function of curcumin in regards to GLP-1 secretion.

Acknowledgments

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