Title

(–)-Epicatechin and Anthocyanins Modulate GLP-1 Metabolism: Evidence from C57BL/6J Mice and GLUTag Cells

Journal

Journal of Nutrition, 151(6)

ISSN

0022-3166

Authors

Cremonini, Eleonora
Daveri, Elena
Mastaloudis, Angela
et al.

Publication Date

2021-06-01

DOI

10.1093/jn/nxab029

Peer reviewed

The Journal of Nutrition
Nutrition and Disease

See corresponding commentary on page 1365.

(–)-Epicatechin and Anthocyanins Modulate GLP-1 Metabolism: Evidence from C57BL/6J Mice and GLUTag Cells

Eleonora Cremonini,[1] Elena Daveri,[1] Angela Mastaloudis,[2] and Patricia I Oteiza[1]

[1]Department of Nutrition and of Environmental Toxicology, University of California, Davis, CA, USA; and [2]Pharmanex Research, NuSkin Enterprise Products, Inc., Provo, UT, USA

ABSTRACT

Background: Generated in intestinal L cells through cleavage of proglucagon (Gcg), glucagon-like peptide 1 (GLP-1) is secreted and rapidly inactivated by dipeptidyl peptidase IV (DPP-IV). GLP-1 regulates insulin secretion and overall glucose homeostasis. The capacity of dietary bioactives to increase GLP-1 circulating levels, and therefore increase insulin secretion and glucose metabolism, has gained significant interest of late.

Objectives: We evaluated the effects of (–)-epicatechin (EC) and different anthocyanins (ACs) and AC metabolites on GLP-1 metabolism in mice and on GLUTag cells.

Methods: We fed 6-week-old C57BL/6J male mice a control diet or a control diet supplemented with either 40 mg AC or 20 mg EC/kg body weight for 14 weeks (AC) or 15 weeks (EC). Intestinal mRNA levels of *Gcg* and *Dpp-iv* were measured. In vitro, GLUTag cells were incubated in the presence or absence of different ACs, the AC metabolite protocatechuic acid (PCA), and EC. GLP-1 secretion and the main pathways involved in its release were assessed.

Results: Long-term supplementation with EC or AC increased mouse GLP-1 plasma concentrations (55% and 98%, respectively; $P < 0.05$). In mice, *1*) EC and AC increased *Gcg* mRNA levels in the ileum (91%) and colon (41%), respectively ($P < 0.05$); and *2*) AC lowered ileum *Dpp-iv* mRNA levels (35%), while EC decreased plasma DPP-IV activity (15%; $P < 0.05$). In GLUTag cells, *1*) cyanidin, delphinidin, PCA, and EC increased GLP-1 secretion (53%, 33%, 53%, and 68%, respectively; $P < 0.05$); and *2*) cyanidin, delphinidin, EC, and PCA increased cyclin adenosine monophosphate levels (25–50%; $P < 0.05$) and activated protein kinase A (PKA; 100%, 50%, 80%, and 86%, respectively; $P < 0.05$).

Conclusions: In mice, EC and ACs regulated different steps in GLP-1 regulation, leading to increased plasma GLP-1. Cyanidin, delphinidin, PCA, and EC promoted GLP-1 secretion from GLUTag cells by activating the PKA-dependent pathway. These findings support the beneficial actions of these flavonoids in sustaining intestinal and glucose homeostasis through the modulation of the GLP-1 metabolism. *J Nutr* 2021;151:1497–1506.

Keywords: (–)-epicatechin, anthocyanins, intestine, L cells, GLP-1, glucose metabolism

Introduction

The intestinal derived hormone glucagon-like protein 1 (GLP-1) has gained particular interest in the past decade because of its capacity to modulate glucose, lipid, and energy metabolisms (1–3). In fact, GLP-1 agonists are currently used in the treatment of type 2 diabetes and other endocrinological disorders (4–7). In addition to its effects on glucose homeostasis, GLP-1 is known to modulate food intake, satiety, and gastric emptying, as well as regulate fluid homeostasis and gastrointestinal tract motility (8). Recently, GLP-1 has been identified as a major signaling molecule in the gut/brain axis, where its pleiotropic effects include thermogenesis, blood pressure control, neurogenesis, and neuroprotection (9). For these reasons, there is currently major scientific interest in understanding its regulation by dietary factors, including phytochemicals.

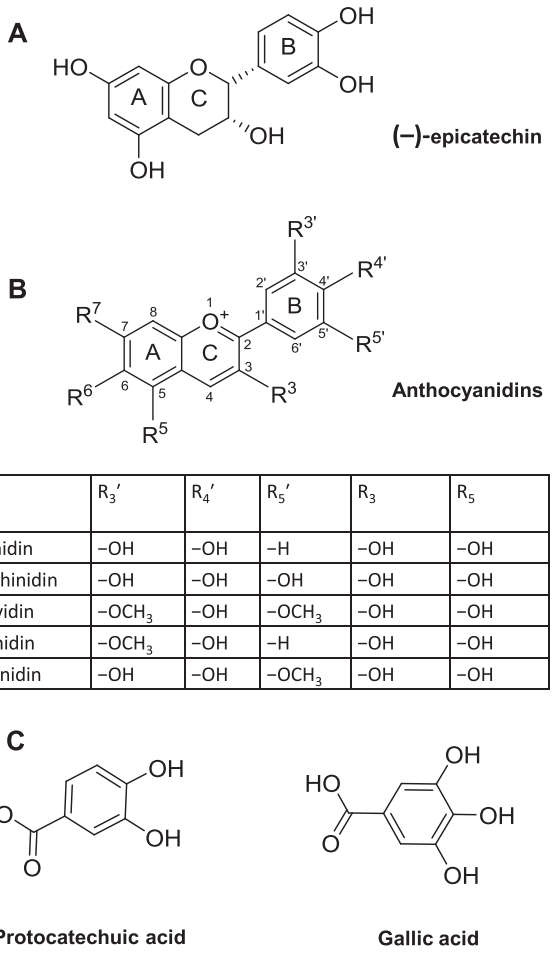
GLP-1 is synthesized in the L-enteroendocrine cells of the gastrointestinal tract. It is synthesized as a precursor peptide [preproglucagon (Gcg)], which is subsequently cleaved by the proteolytic enzyme proprotein convertase 1/3 to generate GLP-1, glucagon-like protein 2 (GLP-2), intervening peptide 2, glicentin, and oxyntomodulin. GLP-1 secretion is mainly stimulated by macronutrient intakes, but also by other molecules, including α and β adrenergic receptor agonists (7, 10). The physiological actions of GLP-1 in target organs are exerted through its binding to the G-protein coupled GLP-1 receptor. Notably, GLP-1 is rapidly cleaved by the enzyme dipeptidyl peptidase IV (DPP-IV), either before the hormone leaves the gastrointestinal tract or after it enters the circulation. Therefore, higher levels of DPP-IV either in the gastrointestinal tract or within the circulation limit GLP-1's half-life and activity.

Manuscript received October 22, 2020. Initial review completed November 18, 2020. Revision accepted January 26, 2021.
First published online March 10, 2021; doi: https://doi.org/10.1093/jn/nxab029.

There is an increasingly strong body of literature indicating that select flavonoids and flavonoid-containing extracts exert antidiabetic and antiobesity effects (11–14). Preliminary evidence suggests that these benefits may, in part, be related to the modulation of GLP-1 synthesis and metabolism. Both in vivo and in vitro studies have provided evidence that select flavonoids increase circulating GLP-1 and promote its release from intestinal enteroendocrine cells (13–16). Such compounds could act by affecting the GLP-1 synthesis, increasing its half-life or its release from L cells. Any of these mechanisms of action would ultimately cause a rise in GLP-1 plasma levels.

All flavonoids consist of a common 3-ring structure (**Figure 1**A and B). Within the flavonoid class, the anthocyanidin family contains double bonds in the 3 rings and a positive charge in the C ring on the oxygen atom (17). Anthocyanidins exist in nature as their glycosylated forms, the anthocyanins (ACs). The different members of the AC family (e.g., cyanidin, delphinidin, malvidin, petunidin, peonidin) are defined by the presence of hydroxyl group substitutions that differ in number and position (Figure 1B). 3,4-Dihydroxybenzoic acid [protocatechuic acid (PCA)] and 3,4,5-trihydroxybenzoic acid (gallic acid) are metabolites of AC consisting of a simple aromatic phenolic ring structure with different hydroxyl group substitutions (Figure 1C). The flavanol (–)-epicatechin (EC) consists of a flavan-3-ol structure and, interestingly, possesses the same distribution of hydroxyl substituents as cyanidin in the B ring (Figure 1A). While ACs are present in nature, and thus in food, as glycosylated compounds, EC exists as an aglycone. Both AC and EC consumption have been demonstrated to modulate GLP-1 and GLP-2 plasma levels in mice. For example, we previously observed that chronic dietary supplementation of C57BL/6J mice with a cyanidin- and delphinidin-rich extract increased plasma levels of GLP-1 and GLP-2 (13, 18). In a similar experimental model, EC increased plasma GLP-2 concentrations; however, GLP-1 was not evaluated (12).

Plasma levels of GLP-1 are increased in EC- and AC-supplemented mice, as currently reported for EC and previously described for AC (13). However, the mechanisms responsible for these increases are unknown. In C57BL/6J mice supplemented with either AC (cyanidin and delphinidin) or EC, this paper characterized the effects of these compounds on various steps in the GLP-1 metabolic pathway. In GLUTag cells, a model of enteroendocrine L cells, we investigated the capacity of EC and different ACs and AC metabolites to modulate the GLP-1 metabolism and GLP-1 release, and characterized the underlying mechanisms responsible for their effects. Results indicate that AC and EC can act at different levels in the GLP-1 metabolism in vivo. In vitro studies point to a structure-dependent effect on the GLP-1 release, as differential effects were observed for individual ACs and AC metabolites. Activation of protein kinase A and increases in intracellular calcium appear to be the main molecular mechanisms underlying EC and AC effects on GLP-1 release.



AC	R_3'	R_4'	R_5'	R_3	R_5
Cyanidin	–OH	–OH	–H	–OH	–OH
Delphinidin	–OH	–OH	–OH	–OH	–OH
Malvidin	$–OCH_3$	–OH	$–OCH_3$	–OH	–OH
Peonidin	$–OCH_3$	–OH	–H	–OH	–OH
Petunidin	–OH	–OH	$–OCH_3$	–OH	–OH

FIGURE 1 Chemical structures of (A) (–)-epicatechin, (B) anthocyanidins, and (C) anthocyanidin metabolites protocatechuic acid and gallic acid. (B, inset table) Different anthocyanidin family members (cyanidin, delphinidin, malvidin, peonidin, and petunidin) are defined on the basis of the presence of hydroxyl group substitutions that differ in number and position. Chemical structures were drawn using ChemDraw Professional 19.0 software (PerkinElmer Inc.).

Methods

Materials

Cell culture media and reagents were from Invitrogen/Life Technologies. Primary antibodies for β-actin (#12,620), phospho–Ca^{2+}/calmodulin-dependent protein II (CaMKII; Thr286; #12,746), CaMKII (#4436), phospho–protein kinase A (PKA) C (Thr197; #5661), PKA (#3927), phospho (Thr202/Tyr204)-p44/42 extracellular signal-regulated kinase $\frac{1}{2}$ (ERK1/2; #4370), p44/42 ERK1/2 (#4695), and U0126 were

This work was supported by the mission of the National Institute of Food and Agriculture-U.S. Department of Agriculture (NIFA-USDA) (CA-D*-NTR-7244-H) and an unrestricted gift from Pharmanex Research, NSE Products Inc., Provo, Utah.

Author disclosures: PIO has received research grants from NSE Products Inc. and is a member of the NSE Products Inc. Advisory Board; has received research grants from other food companies and government agencies with an interest in health and nutrition; and is a correspondent researcher from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

AM was employed by Pharmanex Research, a company that provided financial support and the anthocyanin-rich mix for the mouse study. EC and ED, no conflicts of interest.

Supplemental Tables 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

EC and ED contributed equally to this work.

Address correspondence to PIO (e-mail: poteiza@ucdavis.edu).

Abbreviations used: AC, anthocyanin; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N, N, N′, N′-tetraacetic acid tetrakis (acetoxymethyl ester); CaMKII, Ca^{2+}/calmodulin-dependent protein II; DPP-IV, dipeptidyl peptidase IV; EC, (–)-epicatechin; ERK1/2, extracellular signal-regulated kinase 1/2; Gcg, proglucagon; GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like protein 2; HED, human equivalent dose; KRB, Krebs-Ringer bicarbonate buffer; PCA, protocatechuic acid; PKA, protein kinase A; PKCζ, protein kinase ζ.

from Cell Signaling Technology. Antibodies for α-tubulin (sc-23,948) and protein kinase ζ (PKCζ; sc-393,218) were from Santa Cruz Biotechnology. The enhanced chemiluminescence Western blotting system was from Thermo Fisher Scientific Inc. GLP-1 was determined using a kit from Crystal Chem Inc. The AC-rich mix was provided by NSE Products, Inc., and its composition is described in Daveri et al. (13). We assessed cAMP using an ELISA kit from Abcam. Delphinidin-3-O-glucoside, cyanidin-3-O-glucoside (Cy), petunidin-3-O-glucoside, peonidin-3-O-glucoside, and malvidin-3-O-glucoside were from Extrasynthese (Genay Cedex). Vildagliptin, (–)-epicatechin, gallic acid, protocatechuic acid, KN-93, H-89, and all other chemicals were purchased from Sigma-Aldrich Co.

Animals and animal care

All procedures were carried out in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals; experimental protocols were approved before implementation by the University of California, Davis, Animal Use and Care Administrative Advisory Committee. Procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis.

We purchased 6-week old healthy male C57BL/6J mice (20–25 g; 10 mice/group) from The Jackson Laboratories, and for 14 (AC) or 15 (EC) weeks we fed them either: *1*) a diet containing approximately 10% total calories from fat (control; C group); *2*) the control diet plus 40 mg AC/kg body weight (C + AC group); or *3*) the control diet supplemented with 20 mg EC/kg body weight (C + EC group; diet composition is provided in **Supplemental Table 1**). The descriptions of the diets and the composition of the AC blend used in this study were previously published (12, 13). In early studies, we calculated the amount of EC and AC to supplement using the Reagan-Shaw et al. (19) scaling criteria to relate consumption in humans to mice. Based on this calculation, the human equivalent dose (HED) for 40 mg/kg body weight of AC would be 240 mg, which is slightly lower than the 266 mg AC found in $\frac{1}{2}$ cup (72.5 g) of fresh black currants (20). Similarly, the HED for 20 mg/kg body weight of EC would be 110 mg, which is similar to that found in a 100 g (3.5 oz) bar of dark chocolate (21). Previous dose-response studies used 3 doses for each flavonoid based on the lowest and the highest dose achievable through diet or supplementation, respectively (13, 18). Although the lowest doses were effective in improving glucose homeostasis in mice fed a high-fat diet, the largest doses were also capable of fully restoring glucose tolerance and insulin sensitivity. Based on the relevance of GLP-1 to glucose homeostasis, current determinations were conducted in mice fed EC and AC at 20 and 40 mg/kg body weight, respectively, in order to match the most efficacious doses tested previously.

At the end of each study, blood was collected and mice were euthanized as previously described (12, 13). Plasma GLP-1 concentrations were determined following the manufacturer's guidelines (Crystal Chem Inc.). Tissue samples from the duodenum, ileum, and colon were collected, flash frozen in liquid nitrogen, and then stored at −80°C until further analysis. The overall metabolic profiles, food intake, and body weight of these animals were recently published (12, 13, 18).

GLUTag cell culture

GLUTag cells (a kind donation from Dr. D.J. Drucker, University of Toronto, Toronto, Canada) were cultured at 37°C in a humidified atmosphere with 5% (v/v) CO_2 in DMEM low glucose, supplemented with 10% (v/v) fetal bovine serum and with antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). The medium was replaced every 2 days until 80% confluence. All experiments were performed in serum-free DMEM.

GLP-1 secretion

GLUTag cells were grown in 24-well plates (0.3×10^6) for 2 days. After reaching 80% confluence, cells were starved for 1 hour in glucose-free Krebs-Ringer bicarbonate buffer (KRB; 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 22 mM $NaHCO_3$) containing 0.5% (w/v) fatty acid free BSA. Cells were subsequently incubated with different concentrations of ACs [Cy, delphinidin-3-O-glucoside (Del), malvidin-3-O-glucoside, peonidin-3-O-glucoside, or petunidin-3-O-glucoside], protocatechuic acid (PCA), gallic acid, and EC (0.1–10 μM) in KRB containing 0.5% (w/v) BSA for 2 hours. Plates were centrifuged at 500 × *g* for 3 minutes at room temperature to remove floating cells. The medium was collected and secreted GLP-1 was assessed using an ELISA kit specific for total GLP-1 (1-36), (7-36), and (9-36) amides (Crystal Chem Inc.) following the manufacturers' protocol. The concentration of GLP-1 in the medium was normalized to the cell protein content as measured using the Bradford method (22).

Effects of signaling inhibitors on GLP-1 secretion and intracellular cAMP

After reaching 80% confluence, the GLUTag cells' growing medium was replaced with serum-free DMEM containing specific inhibitors for: CaMKII (KN-93), PKA (H-89), MEK-1,2 (U0126; 20 μM), and intracellular Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM; 10 μM); cells were incubated for 1 hour. Subsequently, EC, AC and metabolites (5 μM each) were added and cells further incubated for 2 hour. Medium GLP-1 concentration was measured as described above. For the determination of intracellular cAMP, cells were incubated with 0.1 M HCl for 30 minutes at room temperature, collected and centrifuged at 600 × *g* for 10 minutes. cAMP was assessed in the supernatant using an ELISA kit (Abcam) following the manufacturers' protocol.

Western blot analysis

GLUTag cells' growing medium was replaced with serum-free DMEM for 1 hour. After incubation, cells were treated with Cy, Del, or PCA (5 μM each) for 2 hours. Cell total homogenates were prepared and Western blots were carried out as previously described (23, 24). The bands were acquired using ChemiDoc Imagers and the intensity of the bands were quantified using ImageLab software (BioRad Laboratories).

RNA isolation and qPCR

For qPCR studies, RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen) following the manufacturers' instructions. The cDNA was generated using high-capacity cDNA Reverse Transcriptase (Applied Biosystems). The mRNA levels of proglucagon (*Gcg*), dipeptidyl peptidase IV (*Dpp-iv*), and proprotein convertase 1/3 (*Pc 1/3*) were assessed by qPCR (iCycler, Bio-Rad) using the primers described in **Supplemental Table 2**. The Ct values were normalized to β-actin. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

DPP-IV activity assay

DPP-IV activity was measured as described in Scharpé et al. (25) using glycyl-prolyl-4-methoxy-β-naphthylamide (Gly-Pro-4-Me-β-NA) as the substrate and Vildagliptin as the negative control (26).

Statistical analysis

Statistical analysis was performed by Statview 5.0 (SAS Institute Inc.). Data from mice and cells were tested for normal bell-shaped curve distribution and variance homogeneity and subsequently analyzed using a Student's *t*-test or 1-way ANOVA to calculate the significant differences among the groups' treatments. Mice intestinal *Gcg* and *Dpp-iv* mRNA levels were analyzed using unpaired Student's *t*-tests. Given that 2 independent animal studies were conducted, C + EC and C + AC were only compared to their respective control group in each study. Plasma DDP-IV activity and the GLUTag cell data were analyzed by a 1-way ANOVA. The GLUTag cells' kinetic data was analyzed by a 2-way ANOVA. Data are presented as the fold of control (fold of C) and are reported as means ± SEMs. A multiple comparison Tukey's post-hoc test was used to examine significant differences between the group means. Labeled means without a common symbol or letter significantly differ. A P value < 0.05 was considered statistically significant.

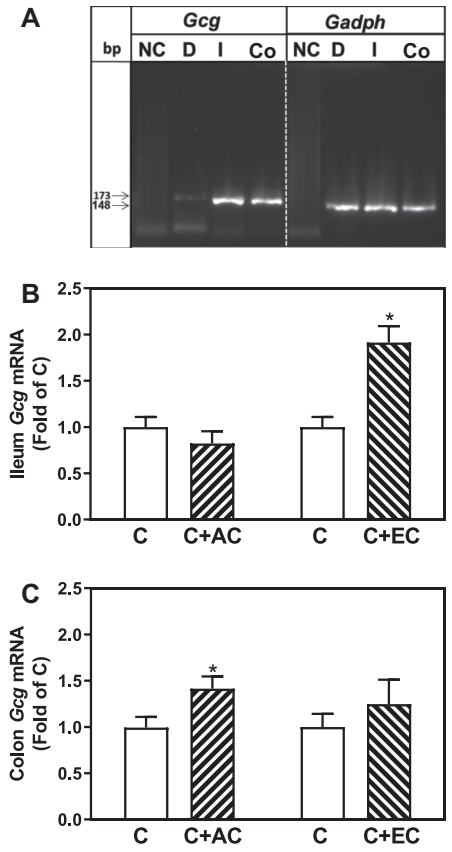


FIGURE 2 (A) Intestinal *Gcg* mRNA abundance and effect of EC and AC supplementation on *Gcg* mRNA levels in the (B) ileum and (C) colon in mice. Data are shown as means ± SEMs of 8–10 animals/group. *Different from control at a P value < 0.05. Abbreviations: AC, anthocyanins; C, control; Co, colon; D, duodenum; EC, (–)-epicatechin; I, ileum; NC-, negative control.

Results

Supplementation with AC and EC affect GLP-1 metabolism in mice

We previously observed that AC supplementation increased plasma levels of GLP-1 in this same cohort of mice (13). Regarding EC, using samples from a previously published study (12), we now report that the plasma GLP-1 concentration was significantly higher ($P < 0.02$) in mice supplemented for 15 weeks with EC (20 mg/kg body weight) compared to control mice (18.7 ± 1.9 and 12.1 ± 1.6 pM, respectively). Furthermore, we assessed the activity and expression of proteins involved in the GLP-1 metabolism in AC- and EC-supplemented mice. High levels of *Gcg* mRNA were expressed in the mouse ileum and colon, but not in the duodenum (**Figure 2**A). While supplementation with the AC blend did not affect *Gcg* mRNA levels in the ileum, it caused a 41% increase in the colon (Figure 2B and C). In contrast, EC supplementation caused a 91% increase in ileum *Gcg* mRNA levels, but did not affect levels in the colon (Figure 2B and C).

GLP-1 is rapidly cleaved and thereby inactivated by the DPP-IV protease. In the ileum, the AC-supplemented group showed 35% lower *Dpp-iv* mRNA levels compared to the control group, while EC supplementation had no effect (**Figure 3**A). *Dpp-iv* mRNA levels in the colon were not impacted by either of the treatments (Figure 3B). In plasma, DPP-IV activity was similar between the control and AC-supplemented groups, while a 20% lower activity was observed in EC-supplemented mice compared to the control group (Figure 3C).

The major components of the AC blend used to supplement the mice were cyanidin, delphinidin, and the cyanidin metabolite PCA (13). Thus, we next investigated the effects of 5 μM cyanidin, delphinidin, PCA, and EC on *Dpp-iv* mRNA levels in GLUTag cells. After 6 hours of incubation, cyanidin, PCA, and EC, but not delphinidin, decreased *Dpp-iv* mRNA levels (by 27%, 12%, and 21%, respectively) when compared to the control, nonadded cells (Figure 3D).

ACs, AC metabolites, and EC differentially affect GLP-1 secretion in GLUTag cells

In GLUTag cells, we initially investigated the capacity of ACs, AC metabolites, and EC to promote GLP-1 release to the medium. Among all the different members of the AC family tested, cyanidin, its metabolite PCA, and delphinidin were the most effective at promoting GLP-1 release after 2 hours of incubation (**Figure 4**A, B, and F); a similar effect was observed for EC (Figure 4H). After 2 hours of incubation, cyanidin and PCA showed a similar concentration-dependent stimulation of GLP-1 release (1–10 μM). At a 5 μM concentration, cyanidin and PCA caused a 53% increase and delphinidin a 33% increase in GLP-1 secretion (Figure 4A, B, and F). Within the range of concentrations tested (0.1–10 μM), malvidin, petunidin, and gallic acid had no effect on medium GLP-1 levels (Figure 4C, E, and G), while peonidin caused a 47% increase in GLP-1 secretion only at a concentration of 10 μM (Figure 4D). Due to the lack of effect of malvidin, petunidin, peonidin, and gallic acid at a concentration of 5 μM on GLP-1 excretion, these compounds were excluded from later experiments. EC was as effective as cyanidin and PCA, causing a 50% increase in GLP-1 secretion at a 5 μM concentration (Figure 4H).

The kinetics of GLP-1 secretion following incubation with 5 μM cyanidin, delphinidin, EC, and PCA are depicted in **Figure 5**A. There were no significant changes in GLP-1 in any group after 1 hour; however, similar increased levels were recorded in all treatment groups after 2 hours. At 6 hours of incubation, although GLP-1 excretion remained significantly higher compared to time 0 in all active compound groups, GLP-1 was significantly higher in the EC and PCA groups compared to the cyanidin and delphinidin groups (Figure 5A). We next investigated whether these increases could be related to changes in the expression of the precursor protein (Gcg), Pc 1/3 (the enzyme responsible for cleaving Gcg to render GLP-1), and DPP-IV. Results showed that cyanidin, delphinidin, PCA, and EC had no effect on mRNA levels of *Gcg*, *Pc 1/3*, or *Dpp-iv* in GLUTag cells after 2 hours of incubation (Figure 5B).

Mechanisms involved in the capacity of cyanidin, delphinidin, PCA, and EC to promote GLP-1 secretion from GLUTag cells

Given that the expression of the main proteins involved in GLP-1 production and degradation were not affected, we next investigated the potential signals that may mediate the capacity of cyanidin, delphinidin, PCA, and EC to promote GLP-1 release from GLUTag cells. Different signaling pathways regulate GLP-1 secretion, which depends on the particular stimulus (27). These signals include the atypical protein kinase C, PKCζ, CaMKII pathway, ERK1/2, and cAMP/PKA. To evaluate the activation of these cascades, we used Western blot to measure the phosphorylation of CaMKII at Thr286, of PKA at Thr197, and of ERK1/2 at Thr202/Tyr204, as well as the expression of PKCζ (**Figure 6**A–D) in response to the active compounds

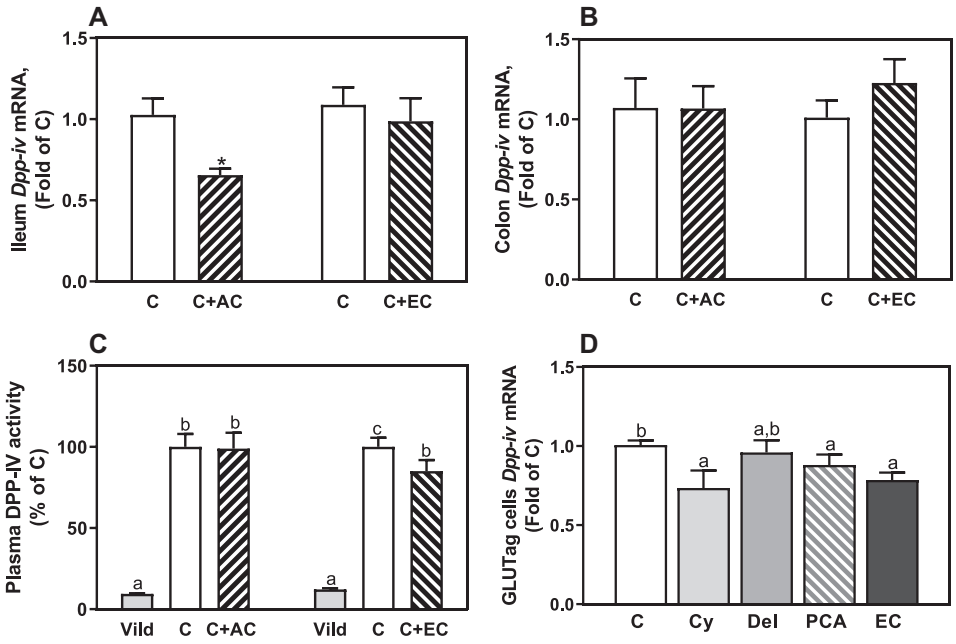


FIGURE 3 EC and AC on DPP-IV expression and activity in mouse (A) ileum, (B) colon, and (C) plasma and in (D) GLUTag cells. Vild was used as a negative control. GLUTag cells incubated for 6 hours in the absence or presence of 5 μM Cy, Del, PCA, or EC. Data are shown as means ± SEMs of (A–C) 8–10 animals/group and (D) 5–6 independent experiments. *Different from control ($P < 0.05$, unpaired *t*-test). Labeled means without a common letter differ at a P value < 0.05. Abbreviations: AC, anthocyanins; C, control; Cy, cyanidin 3-O-glucoside; Del, delphinidin 3-O-glucoside; DPP-IV, dipeptidyl peptidase IV; EC, (–)-epicatechin; PCA, protocatechuic acid; Vild, Vildagliptin.

at 5 μM concentration. Cyanidin and delphinidin promoted small but significant increases (27% and 30%, respectively) in CaMKII phosphorylation compared to control cells, while PCA and EC had no effects (Figure 6A). Cyanidin, delphinidin, PCA, and EC all stimulated PKA phosphorylation (100%, 50%, 80%, and 86%, respectively; Figure 6B) and ERK1/2 phosphorylation (75%, 59%, 78%, and 57%, respectively; Figure 6C). Only cyanidin caused a significant increase in PKCζ protein levels (28% compared to the control group; Figure 6D).

Given the observed increase in PKA phosphorylation, we next investigated whether the tested compounds could affect intracellular cAMP levels. Compared to control cells, cyanidin, delphinidin, and EC caused an approximate 25% increase and PCA a 55% increase in cellular cAMP levels (Figure 6E).

Effects of specific signaling inhibitors on the capacity of cyanidin, delphinidin, PCA. and EC to promote GLP-1 secretion from GLUTag cells

We further investigated the signaling pathways involved in the promotion of GLP-1 release by cyanidin, delphinidin, PCA, and EC using specific inhibitors for CaMKII (KN-93), PKA (H-89), and, to inhibit ERK1/2 phosphorylation, a mitogen-activated protein kinase MEK1/2 inhibitor (U0126). In addition, to assess the involvement of intracellular Ca^{2+}, we used BAPTA-AM, a specific intracellular Ca^{2+} chelator. The capacities of KN-93, H-89, and U0126 to inhibit the respective signaling cascades were initially confirmed by Western blot by measuring CaMKII, PKA, and ERK1/2 phosphorylation after 2 hours of incubation with cyanidin (5 μM; **Figure 7**A). H-89 fully prevented cyanidin-, delphinidin-, PCA-, and EC-mediated GLP-1 secretion, while KN-93 had no effect (Figure 7B–E). U0126 was only effective at inhibiting PCA-mediated GLP-1 release (Figure 7D). BAPTA-AM inhibited the GLP-1 release triggered by cyanidin, delphinidin, PCA, and EC (Figure 7B–E).

Discussion

Previous studies suggest that the capacities of EC and select ACs to improve glucose homeostasis, insulin sensitivity, and lipid metabolism, as well as to sustain gastrointestinal tract health, is due at least in part to their capacities to increase gastrointestinal tract and circulating GLP-1 and GLP-2 levels (12, 18). Here, we report that increases in circulating levels of GLP-1 in mice supplemented with EC or with the ACs cyanidin and delphinidin are the result of differential effects of these flavonoids at various levels in the GLP-1 metabolism. Chronic supplementation with EC led to upregulated expression of the GLP-1 precursor Gcg in the ileum, whereas the cyanidin- and delphinidin-rich extract increased *Gcg* mRNA levels in the colon. Where EC treatment inhibited DPP-IV activity in the circulation, the AC blend decreased its expression in the ileum. In vitro, cyanidin, delphinidin, PCA, and EC all stimulated GLP-1 release from GLUTag cells, mainly through their capacities to activate PKA and cAMP.

Macronutrients, hormones, and neural stimuli induce GLP-1 secretion from intestinal enteroendocrine L cells. Once in the circulation, GLP-1 regulates events in multiple tissues that contribute to glucose control (1–3). For example, GLP-1 stimulates pancreatic insulin synthesis and secretion, inhibits glucagon secretion, and promotes an increase in the β cell number. Additionally, effects of GLP-1 on the central nervous system decrease glycaemia by promoting satiety and delaying gastric emptying (3). Increasing evidence in humans and rodents supports the capacity of dietary EC and ACs to improve glucose homeostasis (13, 28). In rodents fed a high-fat or a high-fructose diet, we observed that supplementation with EC and ACs at the concentrations used in our current study prevented the development of systemic glucose and insulin intolerance, as well as liver and adipose tissue insulin insensitivity (11, 13, 29). Although several mechanisms may be involved in these

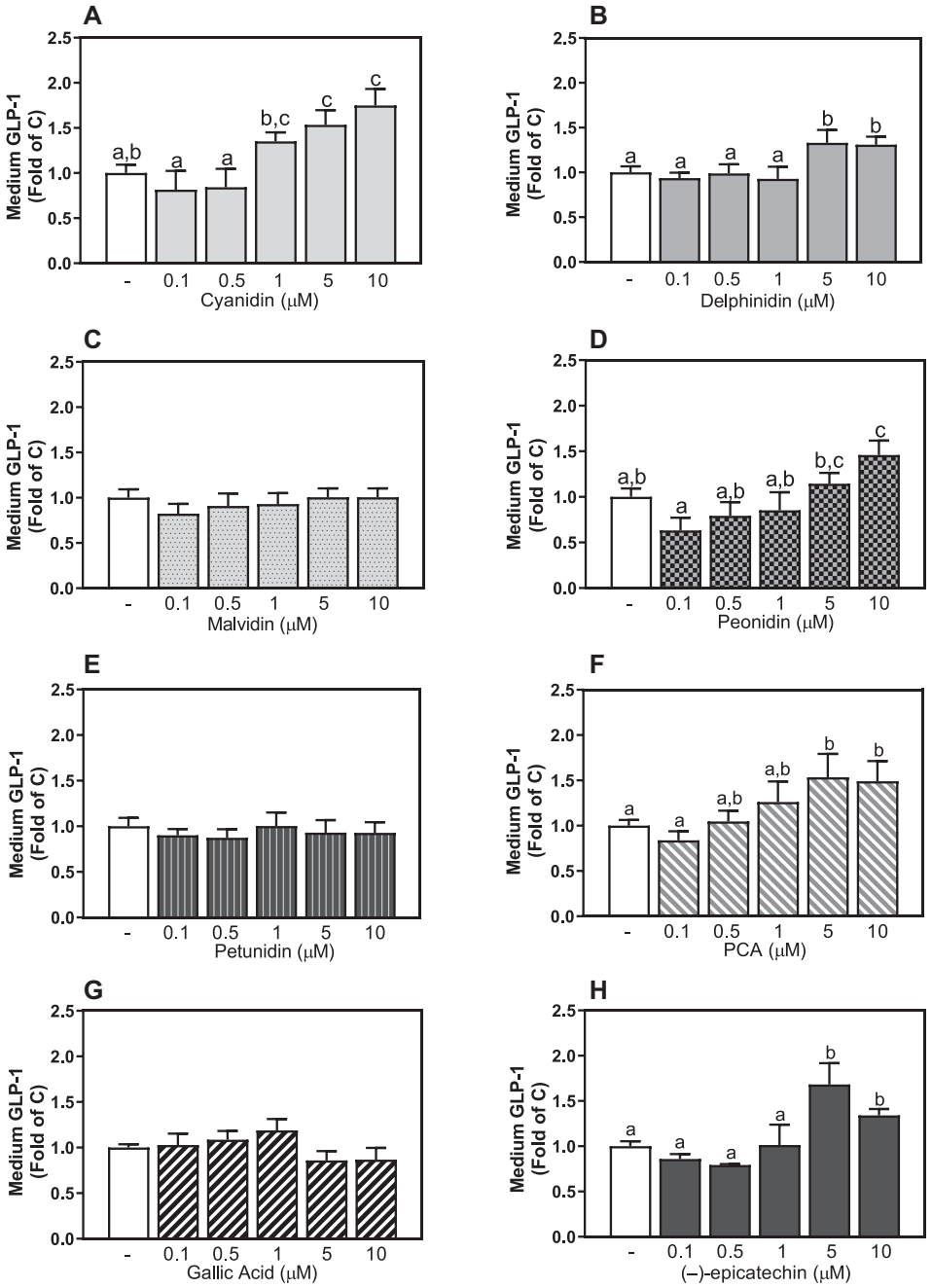


FIGURE 4 The 3-O-glucosides (A) cyanidin, (B) delphinidin, (C) malvidin, (D) peonidin, and (E) petunidin; the AC metabolites (F) PCA and (G) gallic acid; and (H) (–)-epicatechin on GLP-1 secretion from GLUTag cells. Medium GLP-1 concentration in the presence of 0.1–10 μM of the different compounds after 2 hours incubation. Results are shown as means ± SEMs of 5–6 independent experiments. Labeled means without a common letter differ at a P value < 0.05. Abbreviations: C, control; GLP-1, glucagon-like peptide 1; PCA, protocatechuic acid.

protective effects, current evidence suggests that an increase in circulating GLP-1 could be a central mechanism in the regulation of glucose homeostasis by EC and ACs.

In the present study, *Gcg* mRNA intestinal expression was increased by both EC and AC supplementation in mice. Multiple, and still not completely understood, mechanisms regulate Gcg expression. At the level of transcription, different signaling pathways (30) and transactivators [e.g., Cdx-2 (31)] have been identified as regulators of Gcg expression. Regulation through posttranscriptional processing of the *Gcg* mRNA has also been documented (32). Here, AC supplementation caused an increase in *Gcg* mRNA levels in the mouse colon but not in the ileum, while EC supplementation increased levels of *Gcg* mRNA in the ileum, but not in the colon. These differences may be related to the differential absorptions and metabolisms of these flavonoids in different parts of the gastrointestinal tract. EC and ACs per se are not absorbed to any extent. However, EC phase II metabolites are well absorbed in the small intestine, while in contrast there is more limited absorption of AC phase II metabolites. Both EC and ACs are degraded by microbiota in the large intestine, where absorption of the resultant ring fission metabolites takes place (33–35). The products of colonic microbiota metabolism are different for EC and ACs. Valerolactones are the main EC metabolites

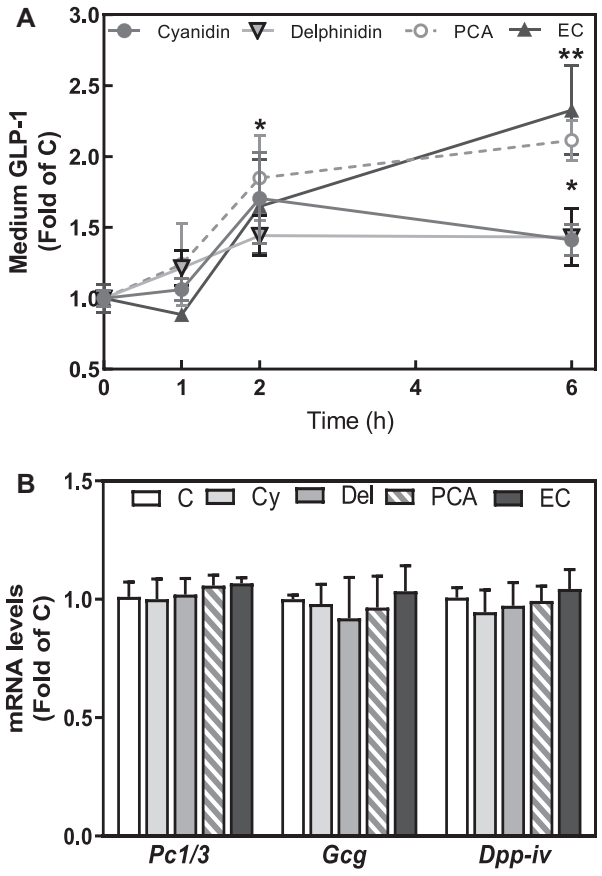


FIGURE 5 (A) Kinetics of GLP-1 secretion and (B) effects of AC, AC metabolites, and EC on events that modulate GLP-1 secretion from GLUTag cells. Cells were incubated for 1, 2, and 6 hours in the presence of 5 μM Cy, Del, PCA, and EC. The mRNA levels of *Pc 1/3*, *Gcg*, and *Dpp-iv* were assessed in cells incubated for 2 hours. Results are shown as means ± SEMs of 5–6 independent experiments. (A) *Significantly different from time 0; **significantly different from other groups at a P value < 0.05. Abbreviations: AC, anthocyanins; C, control; Cy, cyanidin 3-O-glucoside; Del, delphinidin 3-O-glucoside; *Dpp-iv*, dipeptidyl peptidase IV; EC, (–)-epicatechin; Gcg, proglucagon; GLP-1, glucagon-like peptide 1; *Pc 1/3*, proprotein convertase 1/3; PCA, protocatechuic acid.

generated by colonic microbiota. PCA and gallic acid are the principal metabolites generated from cyanidin and delphinidin, respectively, through both microbiota action and spontaneous fission of the ring. In contrast to observations in mice, in GLUTag cells, none of the compounds tested, cyanidin, its metabolite PCA, delphinidin, or EC, affected *Gcg* mRNA levels. The observed in vivo and in vitro differences on the effects of the tested flavonoids/metabolites on *Gcg* mRNA expression may be related to the chronic versus acute type of exposure. Given the complex regulation of Gcg expression, additional studies will be necessary to further understand the mechanisms involved in the upregulation of intestinal Gcg expression by EC and ACs in vivo.

DPP-IV is a widely expressed enzyme that has diverse biological actions. DPP-IV is a membrane protein that, once cleaved and released into the bloodstream in its soluble active form, rapidly cleaves GLP-1, limiting the plasma half-life and activity of GLP-1. Thus, given the relevant function of GLP-1 in sustaining glucose homeostasis, pharmacological inhibitors of DPP-IV are currently used in the treatment of hyperglycemia in type 2 diabetes patients (36). We observed that chronic supplementation with EC decreased DPP-IV activity in mouse plasma. In contrast, while not affecting DPP-IV plasma activity, supplementation with the cyanidin- and delphinidin-rich extract decreased *Dpp-iv* mRNA levels in the mouse colon. Cyanidin, PCA, and EC were all effective at inhibiting DPP-IV expression in GLUTag cells. Thus, the observed effects of EC, select ACs (cyanidin and delphinidin), and AC metabolites on DPP-IV activity and expression can in part explain the increased levels of circulating GLP-1 found in EC- and AC-supplemented mice.

To evaluate the potential mechanisms of action of EC, ACs, and AC metabolites, we used an in vitro model of L cells (GLUTag). GLUTag cells, originated from a mouse intestinal endocrine tumor, are a widely accepted model to assess Gcg synthesis and GLP-1 secretion (30). Various factors can modulate the amount of GLP-1 secreted into the circulation by enteroendocrine cells, including macronutrients, such as carbohydrates and fat, as well as hormones, neural stimuli, and microbiota-derived SCFAs (37, 38). Different receptors and downstream signals activate GLP-1 secretion, depending on the stimulus (38, 39). For example, the sodium-glucose cotransporter 1 triggers voltage-gated Na^+ and Ca^{2+} channel activation, which is required for glucose-mediated GLP-1 intestinal secretion (40). In contrast, PKCζ is involved in oleic acid–induced GLP-1 secretion (41). Other L cell signals found to be involved in GLP-1 secretion include the CaMKII pathway, ERK1/2, and cAMP/PKA (42). Cyanidin, delphinidin, PCA, and EC (1–10 μM) were the most active of the compounds tested in promoting GLP-1 release from GLUTag cells. Consistent with these results, delphinidin 3-rutinoside, but not malvidin 3-rutinoside, was reported to stimulate GLP-1 secretion from GLUTag cells, albeit at a much higher concentration (100 μM) than used in the supplements in the present study (43). We observed that while cyanidin (as cyanidin 3-O-glucoside) activated CaMKII, ERK1/2, and cAMP/PKA and increased PKCζ protein levels, delphinidin activated only CaMKII, ERK1/2, and cAMP/PKA. PCA and EC, in contrast, activated only cAMP/PKA and ERK1/2. For delphinidin-3-O-rutinoside, the effects on GLP-1 release were previously attributed to the activation of the Ca^{2+}-CaMKII pathway (43). In the present experiments, Del at a 10-times lower concentration also activated CaMKII. Interestingly, the inhibition of CaMKII by the inhibitor KN-93 did not affect GLP-1 secretion, suggesting that the increased secretion of GLP-1 by delphinidin is independent of CaMKII pathway activation. Considering the similar levels of GLP-1 secretion stimulation by cyanidin, delphinidin, PCA, and EC, and the complete prevention of this effect by a PKA inhibitor (H-89), their actions seem to be primarily mediated by PKA. These findings are in agreement with evidence that, given their structural similarity to the natural ligand, select flavonoids can stimulate the β-adrenergic receptor and, downstream, PKA (44). However, GLP-1 secretion from GLUTag cells is not increased by β-adrenergic receptor agonists (38), suggesting that other mechanisms may be at play. In β-cells, an increase in intracellular Ca^{2+} activates the PKA pathway with a subsequent increase in insulin secretion (45). In this regard, our results demonstrate that the chemical inhibition of both PKA and intracellular Ca^{2+} chelation by cyanidin, delphinidin, PCA, and EC contributed to the observed increases in GLP-1 secretion in L cells. A lack of action of the CaMKII and ERK1/2 inhibitors suggest these pathways do not play a major role in EC or AC stimulation of GLP-1 secretion. Taken together, these results suggest that the modulation of GLP-1 secretion from L cells by cyanidin, delphinidin, PCA, and EC is

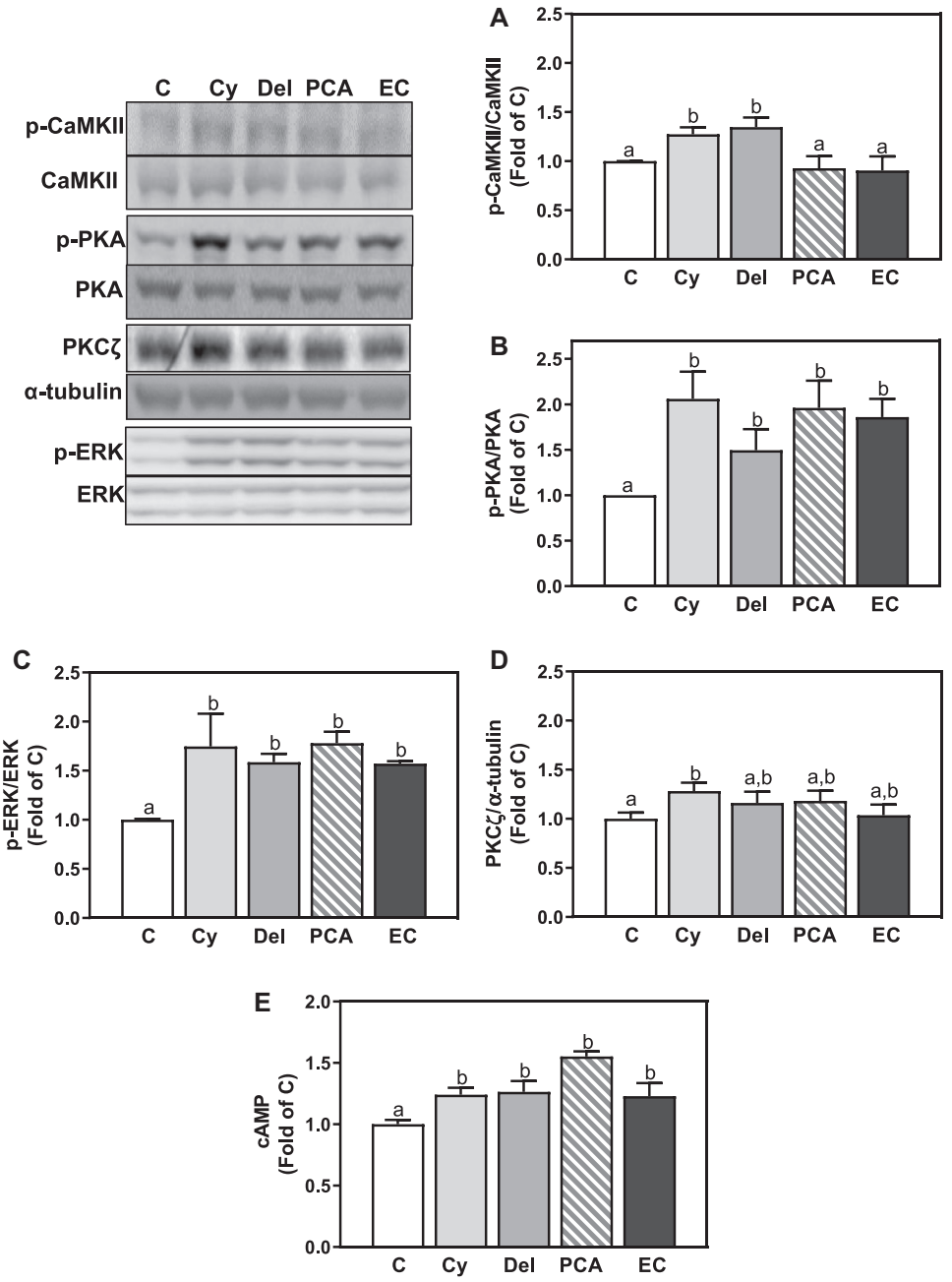


FIGURE 6 Effects of AC, AC metabolites, and EC on (A) phosphorylated CaMKII (Thr286), (B) phosphorylated PKA (Thr197), (C) phosphorylated ERK1/2 (Thr202/Tyr204), (D) PKCζ protein levels, and (E) cAMP cellular levels. Cells were incubated in the absence or presence of 5 μM Cy, Del, PCA, or EC for 2 hours. All results are referred to control values. Results are shown as means ± SEMs of 6–7 independent experiments. Labeled means without a common letter differ at a P value < 0.05. Abbreviations: AC, anthocyanins; C, control; CaMKII, Ca^{2+}/calmodulin-dependent protein II; Cy, cyanidin 3-O-glucoside; Del, delphinidin 3-O-glucoside; EC, (–)-epicatechin; ERK1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated; PCA, protocatechuic acid; PKA, protein kinase A; PKCζ, protein kinase ζ.

mainly dependent on the activation of the cAMP/PKA cascade. This can in part explain the mechanism of action of these flavonoids in the regulation of glucose homeostasis and insulin sensitivity. However, a limitation that should be considered is that we did not include other EC phase II metabolites or microbiota metabolites that are present in the intestine after dietary consumption of the parent compounds. Future studies on EC and AC fecal metabolite concentrations in the intestine and on their distributions among the luminal solid and water mucus phases are needed to better understand their actions and potential interactions.

In summary, EC and select ACs (i.e., cyanidin and delphinidin) increase circulating GLP-1 levels in mice. However, EC and ACs differentially modulate the GLP-1 metabolism. While they all increase *Gcg* expression and downregulate DPP-IV expression and activity, their effects in mice are tissue-specific. Furthermore, Cy, its metabolite PCA, delphinidin, and EC were the most active in promoting GLP-1 excretion through a PKA-dependent mechanism in GLUTag cells. Overall, these results support the concept that the beneficial effects of dietary EC and select ACs at the intestinal level (12, 18) and on systemic glucose and lipid homeostasis (13, 28, 29) can be in part explained

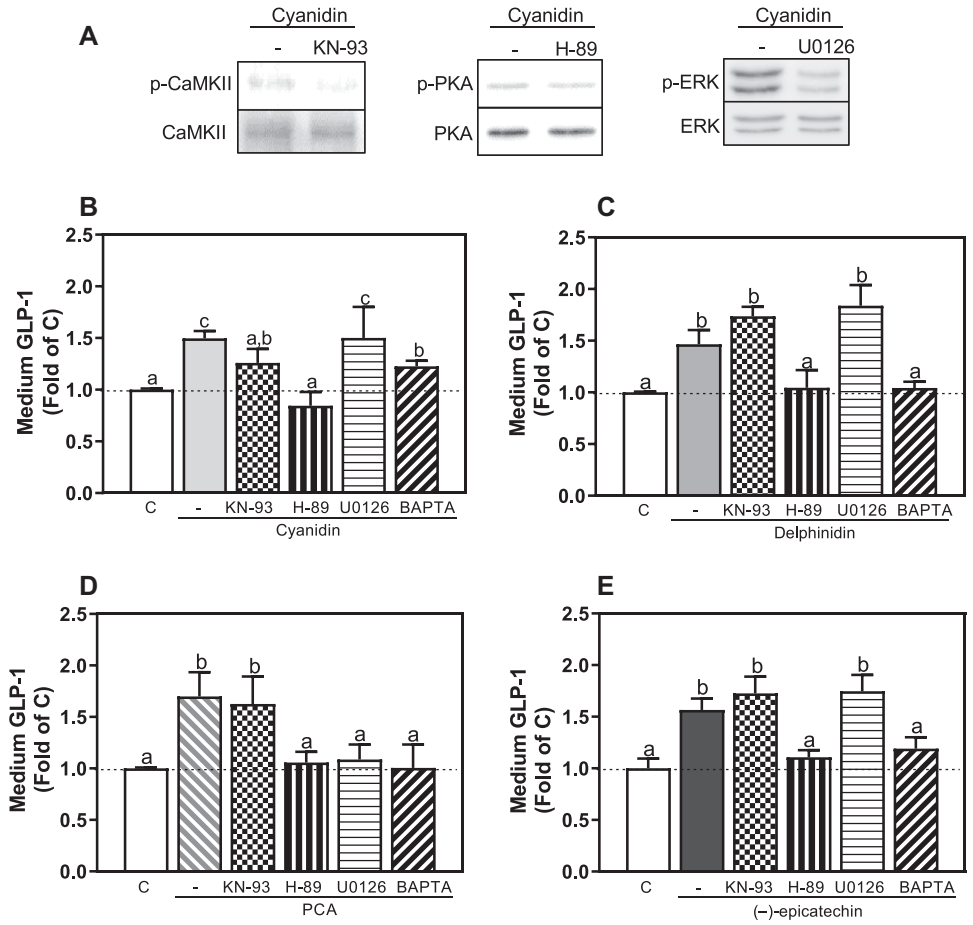


FIGURE 7 (A) Efficacy of the inhibitors assessed and effects of CaMKII, PKA and ERK 1/2 inhibitors and of an intracellular Ca^{2+} chelator on GLP-1 secretion from GLUTag cells induced by (B) cyanidin, (C) delphinidin, (D) PCA, and (E) EC. Efficacy of the inhibitors was assessed by Western blot in cells stimulated with Cy. All results are in reference to control values. Results are shown as means ± SEMs of 6–7 independent experiments. Labeled means without a common letter differ at a P value < 0.05. Abbreviations: BAPTA, 1,2-Bis(2-aminophenoxy)ethane-N, N, N′, N′-tetraacetic acid tetrakis; C, control; CaMKII, Ca^{2+}/calmodulin-dependent protein II; Cy, cyanidin 3-O-glucoside; EC, (–)-epicatechin; ERK1/2, extracellular signal-regulated kinase 1/2; GLP-1, glucagon-like peptide 1; p-, phosphorylated; PCA, protocatechuic acid; PKA, protein kinase A.

by their capacities to modulate GLP-1 homeostasis. A 3'4' catechol group in the B ring seems to be an important chemical structural feature that favors this activity, as ACs that lack this group, such as malvidin and petunidin, had no effect on GLP-1 secretion. Based on the findings that these flavonoids and their metabolites differentially modulate GLP-1 homeostasis, future studies will investigate the synergistic effects of the combination of these putative GLP-1 agonists on glucose regulation and insulin sensitivity.

Acknowledgments

The authors' responsibilities were as follows—EC, PIO, and AM: designed the research; EC and ED: conducted the research and analyzed the data; EC and PIO: wrote the paper and had primary responsibility for the final content; and all authors: edited the manuscript and read and approved the final manuscript.

References

1. Campbell JE, Drucker DJ. Pharmacology, physiology, and mechanisms of incretin hormone action. Cell Metab 2013;17:819–37.
2. Brubaker PL. The glucagon-like peptides: pleiotropic regulators of nutrient homeostasis. Ann NY Acad Sci 2006;1070:10–26.
3. Chia CW, Egan JM. Incretins in obesity and diabetes. Ann NY Acad Sci 2019;1461(1):104–26.
4. Rajeev SP, Wilding J. GLP-1 as a target for therapeutic intervention. Curr Opin Pharmacol 2016;31:44–9.
5. Mishra AK, Dubey V, Ghosh AR. Obesity: an overview of possible role(s) of gut hormones, lipid sensing and gut microbiota. Metabolism 2016;65:48–65.
6. Drucker DJ, Yusta B. Physiology and pharmacology of the enteroendocrine hormone glucagon-like peptide-2. Annu Rev Physiol 2014;76:561–83.
7. Brubaker PL. Glucagon-like peptide-2 and the regulation of intestinal growth and function. Compr Physiol 2018;8:1185–210.
8. Müller TD, Finan B, Bloom SR, D'Alessio D, Drucker DJ, Flatt PR, Fritsche A, Gribble F, Grill HJ, Haberner JF, et al. Glucagon-like peptide 1 (GLP-1). Mol Metab 2019;30:72–130.
9. Grieco M, Giorgi A, Gentile MC, d'Erme M, Morano S, Maras B, Filardi T. Glucagon-like peptide-1: a focus on neurodegenerative diseases. Front Neurosci 2019;13:1112.
10. Lim GE, Huang GJ, Flora N, LeRoith D, Rhodes CJ, Brubaker PL. Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell. Endocrinology 2009;150:580–91.
11. Bettaieb A, Cremonini E, Kang H, Kang J, Haj FG, Oteiza PI. Anti-inflammatory actions of (-)-epicatechin in the adipose tissue of obese mice. Int J Biochem Cell Biol 2016;81:383–92.
12. Cremonini E, Wang Z, Bettaieb A, Adamo AM, Daveri E, Mills DA, Kalanetra KM, Haj FG, Karakas S, Oteiza PI. (-)-Epicatechin protects the intestinal barrier from high fat diet-induced permeabilization:

implications for steatosis and insulin resistance. Redox Biol 2018;14:588–99.
13. Daveri E, Cremonini E, Mastaloudis A, Hester SN, Wood SM, Waterhouse AL, Anderson M, Fraga CG, Oteiza PI. Cyanidin and delphinidin modulate inflammation and altered redox signaling improving insulin resistance in high fat-fed mice. Redox Biol 2018;18:16–24.
14. Gonzalez-Abuin N, Martinez-Micaelo N, Margalef M, Blay M, Arola-Arnal A, Muguerza B, Ardevol A, Pinent M. A grape seed extract increases active glucagon-like peptide-1 levels after an oral glucose load in rats. Food Funct 2014;5:2357–64.
15. Gonzalez-Abuin N, Martinez-Micaelo N, Blay M, Green BD, Pinent M, Ardevol A. Grape-seed procyanidins modulate cellular membrane potential and nutrient-induced GLP-1 secretion in STC-1 cells. Am J Physiol Cell Physiol 2014;306:C485–92.
16. Domínguez Avila JA, Rodrigo García J, González Aguilar GA, de la Rosa LA. The antidiabetic mechanisms of polyphenols related to increased glucagon-like peptide-1 (GLP1) and insulin signaling. Molecules 2017;22:903.
17. Crozier A, Del Rio D, Clifford MN. Bioavailability of dietary flavonoids and phenolic compounds. Mol Aspects Med 2010;31:446–67.
18. Cremonini E, Daveri E, Mastaloudis A, Adamo AM, Mills D, Kalanetra K, Hester SN, Wood SM, Fraga CG, Oteiza PI. Anthocyanins protect the gastrointestinal tract from high fat diet-induced alterations in redox signaling, barrier integrity and dysbiosis. Redox Biol 2019;26:101269.
19. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J 2008;22:659–61.
20. Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J Agric Food Chem 2006;54:4069–75.
21. Rothwell JA, Perez-Jimenez J, Neveu V, Medina-Remón A, M'Hiri N, García-Lobato P, Manach C, Knox C, Eisner R, Wishart DS, et al. Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database 2013;2013:bat070.
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
23. Cremonini E, Mastaloudis A, Hester SN, Verstraeten SV, Anderson M, Wood SM, Waterhouse AL, Fraga CG, Oteiza PI. Anthocyanins inhibit tumor necrosis alpha-induced loss of Caco-2 cell barrier integrity. Food Funct 2017;8:2915–23.
24. Vazquez-Prieto MA, Bettaieb A, Haj FG, Fraga CG, Oteiza PI. (-)-Epicatechin prevents TNFalpha-induced activation of signaling cascades involved in inflammation and insulin sensitivity in 3T3-L1 adipocytes. Arch Biochem Biophys 2012;527:113–8.
25. Scharpé S, De Meester I, Vanhoof G, Hendriks D, Van Sande M, Van Camp K, Yaron A. Assay of dipeptidyl peptidase IV in serum by fluorometry of 4-methoxy-2-naphthylamine. Clin Chem 1988;34:2299–301.
26. Matheeussen V, Lambeir A-M, Jungraithmayr W, Gomez N, McEntee K, Van der Veken P, Scharpé S, De Meester I. Method comparison of dipeptidyl peptidase IV activity assays and their application in biological samples containing reversible inhibitors. Clin Chim Acta 2012;413:456–62.
27. Lim GE, Brubaker PL. Glucagon-like peptide 1 secretion by the L-cell: the view from within. Diabetes 2006;55:S70–S7.
28. Cremonini E, Fraga CG, Oteiza PI. (-)-Epicatechin in the control of glucose homeostasis: involvement of redox-regulated mechanisms. Free Radic Biol Med 2019;130:478–88.
29. Cremonini E, Bettaieb A, Haj FG, Fraga CG, Oteiza PI. (-)-Epicatechin improves insulin sensitivity in high fat diet-fed mice. Arch Biochem Biophys 2016;599:13–21.
30. Drucker DJ, Jin T, Asa SL, Young TA, Brubaker PL. Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. Mol Endocrinol 1994;8:1646–55.
31. Chen L, Wang P, Andrade CF, Zhao IY, Dube PE, Brubaker PL, Liu M, Jin T. PKA independent and cell type specific activation of the expression of caudal homeobox gene Cdx-2 by cyclic AMP. FEBS J 2005;272:2746–59.
32. Gillespie AL, Pan X, Marco-Ramell A, Meharg C, Green BD. Detailed characterisation of STC-1 cells and the pGIP/Neo sub-clone suggests the incretin hormones are translationally regulated. Peptides 2017;96:20–30.
33. Ottaviani JI, Momma TY, Kuhnle GK, Keen CL, Schroeter H. Structurally related (-)-epicatechin metabolites in humans: assessment using de novo chemically synthesized authentic standards. Free Radic Biol Med 2012;52:1403–12.
34. Czank C, Cassidy A, Zhang Q, Morrison DJ, Preston T, Kroon PA, Botting NP, Kay CD. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study. Am J Clin Nutr 2013;97:995–1003.
35. de Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, Kay CD. The pharmacokinetics of anthocyanins and their metabolites in humans. Br J Pharmacol 2014;171:3268–82.
36. Paternoster S, Falasca M. Dissecting the physiology and pathophysiology of glucagon-like peptide-1. Front Endocrinol 2018;9:584.
37. Cani PD, Everard A, Duparc T. Gut microbiota, enteroendocrine functions and metabolism. Curr Opin Pharmacol 2013;13:935–40.
38. Brubaker PL, Schloos J, Drucker DJ. Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line. Endocrinology 1998;139:4108–14.
39. Bodnaruc AM, Prud'homme D, Blanchet R, Giroux I. Nutritional modulation of endogenous glucagon-like peptide-1 secretion: a review. Nutr Metab 2016;13:92.
40. Sun EW, de Fontgalland D, Rabbitt P, Hollington P, Sposato L, Due SL, Wattchow DA, Rayner CK, Deane AM, Young RL, et al. Mechanisms controlling glucose-induced GLP-1 secretion in human small intestine. Diabetes 2017;66:2144–9.
41. Iakoubov R, Izzo A, Yeung A, Whiteside CI, Brubaker PL. Protein kinase Czeta is required for oleic acid-induced secretion of glucagon-like peptide-1 by intestinal endocrine L cells. Endocrinology 2007;148:1089–98.
42. Arifin SA, Paternoster S, Carlessi R, Casari I, Ekberg JH, Maffucci T, Newsholme P, Rosenkilde MM, Falasca M. Oleoyl-lysophosphatidylinositol enhances glucagon-like peptide-1 secretion from enteroendocrine L-cells through GPR119. Biochim Biophys Acta Molecular 2018;1863:1132–41.
43. Kato M, Tani T, Terahara N, Tsuda T. The anthocyanin delphinidin 3-rutinoside stimulates glucagon-like peptide-1 secretion in murine GLUTag cell line via the Ca2+/calmodulin-dependent kinase II pathway. PLoS One 2015;10:e0126157.
44. Kuppusamy UR, Das NP. Potentiation of beta-adrenoceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes. Biochem Pharmacol 1994;47:521–9.
45. Choi SE, Shin HC, Kim HE, Lee SJ, Jang HJ, Lee KW, Kang Y. Involvement of Ca2+, CaMK II and PKA in EGb 761-induced insulin secretion in INS-1 cells. J Ethnopharmacol 2007;110:49–55.