**Identification of a Dual-Action Small Molecule with Potent Anti-diabetic and Anti-obesity Activity**

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# ABSTRACT

Type 2 diabetes (T2D) is one of the fasting growing chronic diseases, caused by insulin resistance and pancreatic β-cell dysfunction. While over thirty medications were approved to treat T2D in the United States, less than one in four patients treated with anti-diabetic drugs achieved the glycemic target. Thus, identifying more effective anti-diabetic drugs is still needed for improving glycemic control in T2D patients. Incretins are gut hormones that possess potent insulinotropic action, which have drawn considerable attention in research and developing treatment strategy for T2D. Specifically, glucagon like peptide 1 (GLP-1), the most important incretin that is secreted from enteroendocrine L-cells in response to food ingestion, plays a vital role in maintaining glycemic homeostasis via potentiating glucose stimulated insulin secretion (GSIS) and promoting pancreatic β-cell proliferation and survival. Therefore, targeting L-cells to induce GLP-1 secretion would be an alternative strategy for treating T2D. The goal of this research was to identify low-cost and safe naturally occurring agents as a primary or adjuvant treatment for T2D. Here, I found that a small molecule, elenolic acid (EA), which was generated in our lab but is also present in mature olive and extra virgin olive oil, dose-dependently stimulated GLP-1 secretion in mouse clonal L-cells and isolated mouse ileum crypts. EA induced a rapid increase in intracellular $[Ca^{2+}]_i$ and the production of inositol trisphosphate in L-cells, indicating that EA activates phospholipase C (PLC)-mediated signaling. Consistently, inhibition of PLC ablated EA-stimulated increase of $[Ca^{2+}]_i$ and GLP-1 secretion in L-cells. In addition, EA-triggered GLP-1 secretion from L-cells was blocked by YM-254890, a $G\alpha_q$ inhibitor. Consistent with our *in vitro* study, a single dose of EA acutely stimulated GLP-1 secretion in mice, accompanied by an improved oral glucose tolerance. Chronic administration of EA restored the impaired glucose and lipid homeostasis in DIO mice, which may be partially due to promoting GLP-1 secretion and reduced hepatic

gluconeogenesis. In addition, EA suppressed appetite, reduced food intake and gastric emptying rate, as well as promoted weight loss in obese mice, demonstrating that it is also an anti-obesity agent. Further, EA treatment reduced lipid absorption, and promoted hepatic fatty acid oxidation, and reversed abnormal plasma lipid profiles in DIO mice. Consistently, EA exerted potent anti-diabetic action in db/db mice, and its blood glucose-lowering effect is comparable with that of liraglutide in blood glycemic control but is better than that of metformin in this overt diabetic model. Collectively, I have identified for the first time, as to the best of our knowledge, that EA could be a dual-action compound that exerts anti-diabetic effects via activation of the GLP-1 mediated metabolic pathway and suppression of hepatic gluconeogenesis, leading to effective control on food intake, body weight gain, and glycemia in T2D mice.

**Key words:** Elenolic acid, GLP-1, hyperglycemia, body weight, food intake, mice.

**GENERAL AUDIENCE ABSTRACT**

Type 2 diabetes (T2D) is one of the fasting growing chronic diseases, which results from insulin resistance and pancreatic β-cell dysfunction. Even though there have been over thirty drugs approved to treat T2D in the United States, less than 25% of patients treated with anti-diabetic drugs achieved the glycemic target. Thus, more effective anti-diabetic drugs are still needed for improving glycemic control in the patients with T2D. Incretins are a group of gut hormones and responsible for over 50% postprandial insulin secretion in humans, which have drawn considerable attention in research and developing treatment strategy for T2D. Specifically, glucagon like peptide 1 (GLP-1), the most important incretin that is secreted from enteroendocrine L-cells in response to food ingestion, plays a vital role in controlling blood glucose via potentiating glucose stimulated insulin secretion (GSIS) and promoting pancreatic β-cell proliferation and survival. Therefore, targeting L-cells to induce GLP-1 secretion would be an alternative strategy for treating T2D. The goal of this research was to identify low-cost and safe naturally occurring agents as a primary or adjuvant treatment for T2D. Here, I found that a small molecule, elenolic acid (EA), which was synthesized in our lab but is also present in mature olive and extra virgin olive oil, dose-dependently stimulated GLP-1 secretion in mouse clonal L-cells and isolated mouse ileum crypts (containing L-cells). Further experiments showed that EA induced a rapid increase in intracellular $[Ca^{2+}]_i$ and the production of inositol trisphosphate (IP3) in L-cells, indicating that EA activates phospholipase C (PLC)-mediated signaling, as IP3 is a direct product of PLC. Consistently, inhibition of PLC ablated EA-stimulated increase of $[Ca^{2+}]_i$ and GLP-1 secretion in L-cells. In addition, EA-triggered GLP-1 secretion from L-cells was blocked by YM-254890, a $G\alpha_q$ inhibitor. In line with the *in vitro* study, a single dose of EA acutely elevated plasma GLP-1 concentration in mice, accompanied by an improved oral glucose tolerance. Chronic administration of EA

restored the impaired glucose and lipid homeostasis in diet-induced obese (DIO) mice, which may be partially due to promoting GLP-1 secretion and reduced hepatic gluconeogenesis. In addition, EA suppressed appetite, reduced food intake and gastric emptying rate, as well as promoted weight loss in the DIO mice, demonstrating that it is also an anti-obesity agent. Further, EA treatment reduced lipid absorption and promoted hepatic fatty acid oxidation, as well as reversed abnormal plasma lipid profiles in the DIO mice. Consistently, EA exerted potent anti-diabetic action in predisposed diabetic mice (db/db), and its blood glucose-lowering effect is comparable with that of liraglutide, a commercial GLP-1 receptor agonist, in blood glycemic control but is better than that of metformin, a widely used first line anti-diabetic drug, in this overt diabetic model. Collectively, I have identified for the first time, as to the best of our knowledge, that EA could be a dual-action compound that exerts anti-diabetic effects via activation of the GLP-1 mediated metabolic pathway and suppression of hepatic gluconeogenesis, leading to effective control on food intake, body weight gain, and glycemia in T2D mice.

**Key words:** Elenolic acid, GLP-1, hyperglycemia, body weight, food intake, mice.

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## DEDICATION

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# LIST OF ABBREVIATIONS

**A**

AgRP: Agouti-related peptide

ALT: Alanine aminotransferase

ANOVA: Analysis of variance

ARC: Arcuate nucleus

ATP: Adenosine triphosphate

AUC: Area under the curve

**B**

BMI: Body mass index

**C**

cAMP: Cyclic adenosine monophosphate

CaSR: Calcium-sensing receptor

CCK: Cholecystokinin

CQA: Caffeoylquinic acid

**D**

D3R: Delphinidin 3-rutinoside

DAG: Diacylglycerol

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DIO: Diet-induced obese

DPP-IV: Dipeptidyl peptidase IV

**E**

EA: Elenolic acid

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinases

EVOO: Extra virgin olive oil

**F**

FA: Fatty acid

FBS: Fetal bovine serum

**G**

G6Pase: Glucose-6-phosphatase

GI: Gastrointestinal

GIP: Glucose-dependent insulinotropic peptide

GLP-1: Glucagon-like peptide 1

GLP-1R: Glucagon-like peptide 1 receptor

GLUT: Glucose transporter

GSGS: Glucose stimulated GLP-1 secretion

GSIS: Glucose stimulated insulin secretion

GTT: Glucose tolerance test

GPCR: G-protein coupled receptor

**H**

HBSS: Hanks balanced salt solution

HFD: High fat diet

HOMA-IR: A homeostatic model assessment of insulin resistance

**I**

IBMX: 3-isobutyl-1-methylxanthine

Ip: Intraperitoneal

$IP_3$: Inositol trisphosphate

IR: Insulin resistance

ITT: Insulin tolerance test

**M**

MUFAs: Monounsaturated fatty acids

**N**

NPY: Neuropeptide Y

**P**

PEP1: Peptide transporter-1

PEPCK: Phosphoenolpyruvate carboxykinase

PLC: Phospholipase C

PKA: Protein kinase A

PKC: Protein kinase C

PKD: Protein kinase D

PIP2: Phosphatidylinositol bisphosphate

POMC: Proopiomelanocortin

PTT: Pyruvate tolerance test

PYY: Peptide tyrosine tyrosine

**Q**

qRT-PCR: Quantitative real-time PCR

**R**

SGLT: Sodium-glucose linked transporter

**S**

SCFAs: Short chain fatty acids

SFA: Saturated fatty acids

**T**

T2D: Type 2 diabetes

Tas1R2/3: Taste receptor type 1 member 2/3

TRPM5: $Ca^{2+}$-sensitive transient receptor potential channel M5

# CHAPTER ONE

## Introduction

### Background

Diabetes is one of the fastest growing chronic diseases in the United States where the incidence of diabetes has been doubled in last five years [1]. Type 2 diabetes (T2D) accounts for approximately 90-95% of all the diagnosed diabetes in the United States, with a high prevalence in middle-aged and senior adults [1]. Insulin resistance and subsequently progressive pancreatic β-cell dysfunction are the two defects causing T2D [2]. Thus, preserving pancreatic β-cell function and promoting insulin sensitivity have always been the targets for preventing and treating T2D [3]. Incretins are a group of gut hormones that have an insulinotropic action, which has drawn widely attention for developing novel therapeutic strategy of T2D [4]. Indeed, incretin accounts for over 50% of postprandial insulin secretion in healthy individuals [5]. Glucagon-like peptide-1 (GLP-1), a well-studied incretin that secreted from enteroendocrine L-cells after food ingestion [6], plays a vital role in glucose homeostasis via potentiating glucose stimulated insulin secretion (GSIS) and promoting β-cell proliferation and survival [7-9]. In addition, GLP-1 delays gastric emptying, induces satiety, and reduces body weight in animal models of obesity and obese men [10, 11]. These effects are largely mediated through the GLP-1 receptor (GLP-1R), a $G\alpha_s$-coupled receptor expressed in many tissues such as β-cells, hypothalamus, heart, etc. There are two forms of bioactive GLP-1 in the circulation, GLP-1 (7-36) and GLP-1 (7-37), which are believed to be equally potent in terms of activation of GLP-1R-mediated biological activity [12]. However, GLP-1 only has a few minutes of half-life in the plasma due to the degradation by dipeptidyl peptidase IV (DPP-IV), a serine protease that cleaves the N-terminal histidine and alanine residues from GLP-1 to form the inactive GLP-1 (9-36) or GLP-1 (9-37) amide, which might be a competitive

antagonist for GLP-1 receptor *in vivo* [13]. GLP-1 is released from L-cell in response to ingestion of macronutrients, including fatty acids [14-16], proteins [17], and carbohydrates [18], as well as dietary fibers [19]. In addition, neurotransmitters and neuropeptides that are released from the enteric nervous system and enteroendocrine cells, such as acetylcholine [20], gamma aminobutyric acid (GABA) [21], and gastrin-releasing peptide [22], have been implicated in the regulation of GLP-1 secretion. However, no therapeutic strategy based on these nutrients or factors has been successfully developed so far for treating T2D.

Naturally occurring agents have been used to treat T2D in the past decades, providing a wide source for anti-diabetic drug discovery. Indeed, metformin, the first line of drug for T2D, was derived from the extract of *Galega officinalis* Linn, a widely used herb to treat diabetes in 1920s [23]. In addition, the first approved incretin mimetic, exendin-4 is a naturally occurring GLP-1 analog, which is derived from the exenatide, originally isolated from the saliva of *Heloderma suspectum* [24, 25]. Olive or olive-related food have been shown to have anti-diabetic effects. Specially, a study showed that olive oil ameliorated hyperglycemia in the patients with T2D [26]. In addition, daily intake of 15-20 g olive oil attenuated the risk of T2D by 16% in humans [26]. However, the mechanism underlying the protective effects of olive or olive oil has not been well studied. Extra virgin olive oil (EVOO) is an unrefined olive oil, and made from pure cold-pressed olive, and thus contains fewer chemicals and free radicals. Indeed, EVOO contained polyphenolic compounds with an average of 435 mg/L of gallic acid equivalents [27], particularly hydroxytyrosol, tyrosol, oleuropein, and elenolic acid (EA), which a phenol derivative. In addition, it was shown that administration of tyrosol (20 mg/kg) attenuated fasting hyperglycemia in streptozotocin-induced insulin deficient rats, which was associated with enhanced activity of hexokinase and glucose-6-phosphate dehydrogenase [28]. Oleuropein-enriched diet was also

found to alleviate hyperglycemia and improve oral glucose tolerance in obese diabetic mice [29]. However, the specific targets for these phenolic compounds to exert the observed effects are unclear, given that their poor bioavailability from dietary intake.

A previous study showed that EVOO-enriched meal greatly increased circulating GLP-1 concentrations with a 2.2-fold increase at 1h after meal intake, and 4.7-fold at 2 h after meal intake [27]. EVOO is rich in monounsaturated fatty acids (MUFA), with an average of 75 g per 100 g EVOO [30]. In addition, MUFA-enriched diets stimulated postprandial GLP-1 secretion in the subjects with insulin resistance [31], which might be associated with the G protein coupled receptor, GPCR 119 [32]. Other than MUFAs, oral administration of 20 mg oleuropein, a main polyphenol in EVOO, increased postprandial GLP-1 secretion in healthy individuals [33]. In this regard, both MUFAs and polyphenols might be the contributor to the stimulatory effect of EVOO on GLP-1 secretion. However, the underlying mechanism of the stimulatory effect of phenolic compounds from EVOO on GLP-1 secretion is largely unknown. The composition of phenolic compounds in olive changes during fruit development and maturation. Specifically, for ripened olive, a bitter-taste compound, oleuropein, is hydrolyzed into EA and hydroxytyrosol [34], and therefore, EA is considered a marker of olive maturation. EA is a small molecule with molecule weight of 242 g/mol, and it is one of the main phenolic derivatives from EVOO, with an average content of 10.4 mg/kg [35]. **However, it has never been investigated, as to the best of our knowledge, whether EA possesses anti-diabetic potential. Therefore, my dissertation research was aimed at investigating whether EA has the anti-diabetic effects via stimulating GLP-1 secretion.**

**Hypothesis**

Targeting GLP-1 has been an effective therapeutic avenue for treating T2D [36]. Our main research goal is **to determine whether EA stimulates GLP-1 secretion and exhibits its anti-diabetic potential.** To test the hypothesis, I propose the following specific aims:

**Aim 1: Examine the stimulatory effect of EA on GLP-1 and underlying mechanism *in vitro*.**

Mouse clonal L-cell line and primary ileum crypts will be used to assess the stimulatory action of EA on GLP-1 secretion. Once the stimulatory effect of EA is determined, I will investigate the molecular signaling that modified by EA, of which intracellular $Ca^{2+}$ concentration, protein kinase activity, and phospholipase activity will be assessed.

**Aim 2: Examine the anti-diabetic effect of EA *in vivo*.**

Diet-induced obese mice will be used to assess the anti-diabetic and metabolic effects of EA. In that regard, mice will be given EA via oral gavage to determine the maximal effects of EA on glucose homeostasis, food intake regulation, body weight control, and insulin sensitivity. In addition, body composition, related hormones, and lipid profiles in diabetic mice will be assessed.

**Aim 3: Determine the anti-diabetic efficacy of EA with metformin and liraglutide, in db/db mice.**

Metformin is a first-line oral anti-diabetic drugs that prescribed to the patients in T2D [37]. It is well established that metformin targets liver where it suppressed hepatic glucose production and ameliorated fasting hyperglycemia [38]. Liraglutide is an injectable anti-diabetic drug and shares 97% amino acid sequence identify with human GLP-1 [39]. In this experiment, I will use genetically predisposed diabetic (db/db) mice to assess the anti-diabetic and metabolic effects of EA, as compared to that of metformin and liraglutide. In that regard, mice will be given EA, metformin via oral gavage and liraglutide via subcutaneous injection to determine the maximal

effects of the tested compounds on glucose homeostasis, food intake regulation, body weight control, and insulin sensitivity. In addition, body composition and related hormone profiles in diabetic mice will be assessed.

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# CHAPTER TWO

## Literature Review

**Abstract**

Type 2 diabetes (T2D) results from insulin resistance and dysfunction of pancreatic β-cells. Current consideration of anti-diabetic therapy is focused on improving glucose tolerance or insulin sensitivity of peripheral tissues. Polyphenols, the secondary metabolites from plants, exhibited anti-diabetic effects. While there are limited studies on the effects of polyphenols on humans with T2D, a myriad of studies using rodent models have found that some polyphenolic compounds ameliorate hyperglycemia by improving insulin sensitivity. However, the main targets and locations for polyphenolic compounds to exert anti-diabetic action are unclear. As the first organ exposed to dietary nutrients, gastrointestinal tract is increasingly recognized to play a significant role in regulating glucose homeostasis, which may involve various gut hormones as well as gut microbiota. This chapter reviews the effects of polyphenols in the gut that may be primarily responsible for their anti-diabetic effects. I first provide an overview of the nutrients and glucagon-like peptide 1 (GLP-1) secretion, and summarize the signaling pathways activated by polyphenols in the regulation of the GLP-1 secretion involved in metabolic homoeostasis, with emphasis on recent developments in the effects of phenolic compounds on gut hormones and microbiota as they relate to energy metabolism and glucose homeostasis.

**Key words:** Polyphenol, GLP-1, microbiota, type 2 diabetes, and obesity

## 1 Introduction

Type 2 diabetes (T2D) is becoming a global health problem in humans, with an estimate of 422 million diabetic patients in 2014 [1]. It is affecting more than 30 million or 9% people in the U.S. [2], with 90% of them being overweight or obese [3]. It is well established that T2D result from insulin resistance and chronic pancreatic β-cell dysfunction [4]. Obesity engenders insulin resistance [4] as a result of excess fat accumulation in insulin target organs, especially the liver, skeletal muscle, and adipose tissues, which results in the disruption of the insulin signaling pathway [5]. Most of the therapeutic interventions for T2D focus on lowering blood glucose level by stimulating insulin secretion, decreasing hepatic glucose production, or enhancing insulin action at target tissues [6, 7]. Gastrointestinal (GI) tract plays a critical role in regulating glucose homeostasis, particularly postprandial blood glucose levels. In addition to digesting and absorbing nutrients, the intestine also secretes various hormones as well as generating neuronal signals in response to ingested nutrients that in turn regulate appetite, stomach emptying, gastrointestinal motility, and pancreatic hormone secretion, which collectively exert profound metabolic effects [8-11]. In addition, gut microbiota, which is a large and diverse microbial community in human colon, is involved in metabolizing dietary components, primarily indigestible carbohydrates and phenolic compounds [12]. There is growing evidence showing that the gut microbiota may play an important regulatory role in overall metabolic homeostasis in the host, as alterations in the gut microbiota, termed dysbiosis, are associated with obesity and the pathogenesis of T2D [13, 14].

A growing body of literature from *in vitro* and *in vivo* studies provide evidence that dietary polyphenols, which are present in many fruits, vegetables, and medicinal herbs, may exert anti-diabetic effects [15-18]. It was also found in a recent epidemiological study that a higher intake of polyphenol-enriched diet is associated with a lower risk of diabetes [19]. For example, daily

administration of apple polyphenols for 12 weeks significantly improved glucose tolerance in humans [20]. While various potential mechanisms for the anti-diabetic effects of polyphenolic compounds have been proposed, their anti-diabetic actions are largely unclear. For example, naringenin, a predominant flavanone from grapefruit, was found to increase glucose uptake by cultured skeletal muscle cells [21], but suppress intestinal glucose absorption [22]. Similarly, flavonol quercetin increases glucose uptake in muscle cells but inhibits glucose uptake in adipocytes and intestinal cells [23-25]. In these studies, the lowest effective concentrations of these polyphenolic compounds for achieving the observed results are 25 μM. However, the circulating concentrations of most polyphenols, including their metabolites following dietary intake, are less than 5 μM. Therefore, the physiological relevance of many *in vitro* findings from using pharmacological doses of polyphenols are unknown. Given the low bioavailability but relatively high concentrations of polyphenols in luminal side of GI tract, it is likely that the intestine might be a primary site for polyphenols to exert the observed metabolic actions [26]. Indeed, a large body of literature showed that polyphenols affect glucose and fatty acid digestion and absorption, gut hormone release, and microbiota composition that relate to nutrient digestion and glucose homeostasis [27]. This review will discuss the anti-diabetic actions of polyphenolic compounds with focuses on their effects in the GI tract that may be primarily ascribed to their anti-diabetic effects via gut hormones, glucagon-like peptide 1 (GLP-1), in particular. I first provide an overview of the nutrients and GLP-1 secretion and summarize the signaling pathways activated by polyphenolic compounds in the regulation of the GLP-1 involved in metabolic homoeostasis, with emphasis on recent developments in the effects of phenolic compounds on gut hormones and microbiota as they relate to energy metabolism and glucose homeostasis.

## 2 Nutrients and GLP-1 secretion

### *2.1 GLP-1 physiology*

Gut is the largest endocrine organ in the body and releases an array of gut hormones, which participate in physiological and metabolic regulation [28]. Specifically, meal-induced gut hormone secretion plays a critical role in the regulation of appetite, food intake, and glucose homeostasis [11]. Glucagon-like peptide 1 (GLP-1) is a well-studied incretin hormone, which is primarily secreted by L-cells [29], the second largest population of enteroendocrine cells [30, 31]. GLP-1 is produced from post-translational processing of the proglucagon gene in L-cells [32], leading to the generation of two major forms that are released into the bloodstream, GLP-1-(7–37) and GLP-1-(7–36)$NH_2$ [33]. However, GLP-1 has a very short half-time (2-3 minutes) in blood attributable to the degradation by dipeptidyl peptidase-IV (DPP-IV), which cleaves GLP-1 from its N-terminal into GLP-1-(9-37) and GLP-1-(9-36) $NH_2$ [29]. L-cells are rare in the duodenum and jejunum but primarily located in the ileum and the colon, The polarized morphology of L-cells allows its apical surface to face the lumen of the intestine, which facilitates the cells to sense nutritional, neural, and hormonal signaling [34, 35]. GLP-1 plays a vital role in maintaining glucose homeostasis. It acts via binding to its receptor (GLP-1R), triggering cAMP signaling to augment glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells while inhibiting glucagon release from α-cells [36]. In addition, activation of GLP-1 signaling promotes β-cell proliferation and survival, thereby preserving islet mass [37]. Furthermore, GLP-1 released in response to nutrients reduce gastric emptying rate and gut mobility, and it also targets its receptor in the hypothalamus to promote satiety, thereby suppressing food intake [38, 39].

The impaired GLP-1 secretion in response to nutrients has been reported in individuals with metabolic diseases. Patients with T2D exhibited a significantly lower fasting GLP-1

concentrations as compared with those in healthy subjects [40]. Further, GLP-1 secretion in response to oral glucose intake was significantly attenuated in obese and T2D individuals [41-43]. However, intravenous infusion of GLP-1 in T2D patients reduces fasting and postprandial plasma glucose levels compared with placebo group [44]. Thus, targeting and activating the GLP-1/GLP-1R signaling system could be an effective therapeutic strategy for T2D. Indeed, several GLP-1R agonists have been approved by FDA for treating T2D. The first GLP-1R agonist, exenatide, also known as Byetta, shared 55% homology to human GLP-1, and it attenuated hyperglycemia in T2D patients with the dose of 5 μg [45]. Another GLP-1R agonist, liraglutide, which has 96% homology with human GLP-1, showed more efficacious glycemic control as compared to exenatide [46].

### *2.2 Neuronal and hormonal regulation of GLP-1 secretion*

GLP-1 secretion is primarily stimulated by ingested nutrients, and it has been observed that GLP-1 is secreted into the circulation in a biphasic pattern, with an initial rise of circulating GLP-1 levels occurring 15-30 minutes after food ingestion, followed by a prolonged second phase about 60 minutes later [47]. The early rise of GLP-1 levels following a meal is most likely triggered by neuroendocrine regulators, because the digested nutrient are unable to reach the ileum or colon within 30 minutes where L-cells are primarily located [48]. Indeed, it was found that nutrients in the proximal intestine, known as proximal nutrients, are able to trigger GLP-1 secretion from distal gut via activation of vagal nerves [49, 50]. In the rat GI tract, vagal innervation includes hepatic branch of the part duodenum, and celiac branches of the jejunum, ileum, cecum, and entire colon [51]. A study showed that hepatic branch vagotomy significantly suppressed GLP-1 secretion in rats [49]. Acetylcholine, a well-known neurotransmitter, is involved in the vagal nerve-induced GLP-1 secretion [52]. The receptors of acetylcholine, muscarinic receptors, are expressed on the L-cells, and acetylcholine acts through its receptor to induce GLP-1 secretion [53]. It was reported

that two types of muscarinic receptors on the L-cell membrane are involved in the GLP-1 secretion, M1 and M2 muscarinic receptors. Both of them are G-protein-coupled receptors, although they have different intracellular signaling pathways. Specifically, activation of M1 muscarinic receptor triggers the dissociation of $G\alpha_{q/11}$ subunit and subsequently activates phospholipase C (PLC), which cleaves membrane-associated phosphatidylinositol bisphosphate (PIP2), generating inositol trisphosphate ($IP_3$) and diacylglycerol (DAG). Intracellular $IP_3$ binds to its receptors on the endoplasmic reticulum and then leads to the release of $Ca^{2+}$ and subsequent GLP-1 release. However, activation of M2 muscarinic receptor may induce GLP-1 secretion via the $G\alpha_i$-dependent downstream signaling enzyme, and thereby inhibits adenylyl cyclase and thus attenuates cAMP signaling pathway [54]. In this regard, GLP-1 secretion in response to the activation of M2 muscarinic receptor might suggest that the M2 muscarinic receptor-activated signaling suppressed the inhibitory tonus in the L-cell [54].

GLP-1 secretion is also regulated by gut hormones. Glucose-dependent insulinotropic peptide (GIP), which is secreted from the small intestinal K-cells in response to nutrient intake [29], may also be involved in the regulation of GLP-1 secretion. GIP-induced GLP-1 secretion might at least partially depend on the activation of vagal nerve. Indeed, administration of GIP at a supraphysiological dose to rats increased GLP-1 release, but this effect was abrogated by hepatic branch vagotomy [49]. In addition, GIP receptor is found in L-cells, suggesting that GIP may also directly stimulate GLP-1 release via GIP receptors. GIP stimulated GLP-1 secretion in a dose-dependent manner in fetal rat intestinal cells and GluTag L-cells [55]. In addition to GIP, another gut hormone, ghrelin, which is an orexigenic hormone mainly released from the stomach, was reported to enhance postprandial GLP-1 secretion. The plasma concentration of ghrelin increases during fasting and decreases after refeeding [56]. In clonal L-cells, ghrelin triggered GLP-1

secretion via activation of ghrelin receptor and extracellular signal-related kinase 1/2 (ERK1/2)-dependent pathway [57]. *In vivo*, intraperitoneal administration of ghrelin enhanced glucose stimulated GLP-1 secretion and improved glucose tolerance in mouse, while pharmacological inhibition of ghrelin receptor blocked ghrelin-induced GLP-1 secretion [58]. Consistently, ghrelin-induced improvement in glucose tolerance was diminished in mice administered a GLP-1R antagonist as well as in GLP-1R knockout mice [58], which suggests that the effect of ghrelin on glucose tolerance is mediated through activation of the GLP-1/GLP-1R pathway. Such an interaction between ghrelin and GLP-1 secretion in response to fasting and refeeding demonstrates a precise and reciprocal regulation of food intake in the body. In that regard, increased ghrelin secretion during fasting stimulates appetite and thus increases food intake. The ingestion of food then stimulates GLP-1 secretion, which in turn reduces appetite and decreases ghrelin secretion [59].

### *2.2 Carbohydrates and GLP-1 secretion*

As discussed above, GLP-1 is secreted in a biphasic pattern in response to food intake. The second phase of GLP-1 secretion is primarily stimulated by the digested macronutrients, including carbohydrates, fatty acids, and proteins [60, 61]. The mechanism underlying nutrient-stimulated GLP-1 secretion is summarized as Figure 1. Monosaccharides, like glucose, stimulate GLP-1 secretion *in vitro* and *in vivo* [62, 63]. Glucose is transported by two different transporters in the small intestine, sodium dependent glucose transporter-1 (SGLT1) and glucose transporter-2 (GLUT2). Even though the number of L-cells in the duodenum is low, glucose directly activates duodenal L-cells to induce GLP-1 secretion as demonstrated by intraduodenal glucose infusion in human duodenum [64]. Similarly, glucose induced GLP-1 secretion in the ileum [65], but the

blockage of SGLT1 by phlorizin diminished glucose-stimulated GLP-1 secretion (GSGS) in human ileum [64]. Glucose is transported into L-cells from the lumen and undergoes glycolysis and oxidation, which leads to the production of ATP and therefore increases intracellular ATP/ADP ratio that subsequently results in the closure of $K_{ATP}$ channels, which sequentially causes depolarization of the plasma membrane, opening of voltage-gated L-type $Ca^{2+}$ channels, influx of $Ca^{2+}$ influx, and ultimate GLP-1 secretion [66, 67]. Therefore, inhibition of glucose uptake, then ATP production, the opening of $K_{ATP}$ channels, or closure of $Ca^{2+}$ channels ablated glucose-induced GSGS in human ileum [64]. However, GSGS was not altered in GLUT2 knockout mice [68], suggesting that GSGS is not GLUT2-dependent. Glucose may also stimulate GLP-1 secretion via the glycolysis-independent mechanism. Glucose is a ligand of sweet taste receptors, primarily taste receptor type 1 member 2 (Tas1R2) and taste receptor type 1 member 3 (Tas1R3) [69]. Sweet taste receptors are present in the GI tissue of both rodents and humans [69, 70]. Activation of these receptors by glucose on L-cells triggers GLP-1 secretion via the PLC/$IP_3$/$Ca^{2+}$-sensitive transient receptor potential channel M5 (TRPM5) pathway [71]. Consistently, blockage of Tas1R2/Tas1R3 effectively reduced the increased plasma GLP-1 concentrations after glucose administration in humans [72]. Taken together, glucose stimulated GLP-1 secretion might be mediated by the glucose transporter, SGLT1, or taste receptors, but the preference of glucose is largely unknown.

### *2.3 Fat regulation of GLP-1 secretion*

Fat stimulates GLP-1 release from intestinal L-cells. It has been demonstrated that monounsaturated fatty acids (MUFA), specifically long chain MUFAs, are potent GLP-1 secretagogues, whereas saturated fatty acids (SFA) are not stimulatory [73]. FA-stimulated GLP-1

secretion is primarily mediated through their plasma membrane receptors. Four FA receptors, FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), and FFAR4 (GPR120), which are all coupled to GTP-binding proteins, largely mediate various FA-induced GLP-1 secretion [60, 61]. GPR40 is the receptor for long-chain FAs (LCFAs), such as linoleic acid [74, 75]. FA binding to the receptor activates $G\alpha_q$, which then stimulates PLC to cleave membrane-associated phosphatidylinositol bisphosphate (PIP2) into $IP_3$ and diacylglycerol (DAG). The released $IP_3$ binds to the $IP_3$ receptor on the endoplasmic reticulum (ER), triggering $Ca^{2+}$ release from the ER and subsequent GLP-1 secretion [76]. In addition, DAG can also induce GLP-1 secretion through activation of the protein kinase C (PKC)/protein kinase D (PKD) pathway [77]. It was reported that linoleic acid also binds to FFAR4, the receptor for omega-3 polyunsaturated FAs [78], leading to $G\alpha_q$-coupled $[Ca^{2+}]_i$ increase and GLP-1 secretion [60]. FFAR2 and FFAR3 are activated by short chain FAs (SCFAs), in particular acetate, propionate, and butyrate [61, 79]. Most SCFAs are generated from microbial fermentation of nondigestible dietary nutrients, like fiber and indigestible carbohydrates [80]. FFAR2 is coupled to both $G\alpha_i$ and $G\alpha_q$, whereas FFAR3 is only coupled to $G\alpha_i$ [81]. Activation of FFAR2 triggers the elevation of cytosolic $[Ca^{2+}]_i$ and the phosphorylation of ERK1/2 [61, 81]. Activation of FFAR2 and FFAR3 by propionate resulted in $G\alpha_i$-mediated suppression of intracellular production of cAMP *in vitro* [81], suggesting a potential inhibitory action on GLP-1 secretion. However, mice deficient of FFAR2 or FFAR3 mice displayed impaired GLP-1 secretion in response to administration of SCFAs [61]. In contrast to the results from several previous studies, more recent work using the perfused rat colon demonstrated that specific activation or blockage of FFAR2 and FFAR3 had no effect on colonic GLP-1 release, suggesting that SCFAs trigger GLP-1secretion independent of the FFAR2 and

FFAR3 [82]. Therefore, it remains to be determined whether L-cell-specific FFAR2/FFAR3 are involved in mediating SCFA-stimulated GLP-1 secretion.

### *2.4 Proteins and GLP-1 secretion*

It has been shown that ingestion of high-protein diets induced GLP-1 secretion in rodents and humans [83, 84]. The underlying mechanism of protein-induced GLP-1 secretion might be owing to its enzymatic products, peptides, and amino acids. Specifically, peptide transporter-1 (PEPT1) and calcium-sensing receptor (CaSR) are found to mediate peptide or amino acid-induced GLP-1 secretion [85]. For example, the dipeptide glycine-sarcosine was found to stimulate GLP-1 secretion in primary ileum crypts via a PEPT1-dependent manner [85]. PEPT1 has a relatively broad substrate specificity, as it has been shown that it can transport the di/tripeptides that contain L-amino acids, an acidic or hydrophobic group at C-terminus, a weakly basic group in α-position at N-terminus, a ketomethylene or acid amide bond, or a trans conformation of peptide bonds [86, 87]. In addition to PEPT1, CaSR is involved in the peptide-induced GLP-1 secretion. CaSR is a G-protein-coupled receptor, which is expressed widely in human tissues. In the intestine, CaSR might be involved in the nutrient sensing [88] and serves as a broad-spectrum of L-aromatic amino acid sensor [89]. For example, addition of CaSR inhibitor abrogated the GLP-1 secretion that was induced by L-phenylalanine and L-tryptophan [90, 91]. The activation of CaSR triggers depolarization of the cell membrane that leads to the opening of the voltage gated $Ca^{2+}$ channel and subsequent influx of $Ca^{2+}$ [92]. Other receptors are also involved in amino acid-induced GLP-1 secretion. For example, L-ornithine stimulates the increase in $[Ca^{2+}]_i$ and GLP-1 secretion via the GPRC6A-$G\alpha_q$ pathway, which leads to the activation of PLC and elevation of $[Ca^{2+}]_i$ [93]. However, L-glutamine-induced $[Ca^{2+}]_i$ increase and GLP-1 secretion are PLC-independent, but are

due to the increased intracellular cAMP [67], which suggests that L-glutamine-induced GLP-1 secretion is not associated with CaSR. Interestingly, it was reported that $Na^+$-dependent amino acid transporter, SLC38A2, is involved in the glutamine uptake into L-cells [94]. In addition, intracellular glutamine enhances ATP production via promoting citric acid cycle mediated by glutamate dehydrogenase [95], which might lead to the depolarization of cell membrane, thus, triggering the opening of the voltage gated $Ca^{2+}$ channel and subsequent influx of $Ca^{2+}$ and GLP-1 secretion.

## 3 Polyphenols

Polyphenolic compounds are a group of secondary metabolites that are ubiquitously present in many plants and foods. The biosynthesis of some polyphenols in the plant originates from phenylpropanoid pathway using phenylalanine or shikimic acid as intermediates. Polyphenols are classified on the basis of their diversity of chemical structures as phenolic acids, flavonoids, stilbenes, and lignans [96]. The representative chemical structure of each category is shown in Figure 2. The type and distribution of polyphenols has been well reviewed [97, 98]. Phenolic acids are a group of compounds with a phenolic ring and carboxylic acid. Hydroxycinnamic acids are the most common phenol acids in the fruits [99], which primarily consist of caffeic acid, chlorogenic acid, and ferulic acid. Flavonoids are the largest group of polyphenols. The main structure of flavonoids is diphenylpropanes, consisting of two aromatic rings (A and B) linked by a heterocyclic ring C [100]. Based on the structure of heterocyclic rings, flavonoids are further divided into six groups, flavones, flavonols, flavanones, isoflavones, flavanols, and anthocyanins. One of the well-known stilbenes from human diets is resveratrol, which is present in red wine, grape skin, and blueberry. Lignans are derived from amino acid

phenylalanine via dimerization of substituted cinnamic alcohols. Flax seed and sesame seed are very rich sources of lignans with matairesinols and secoisolariresinols being the dominant forms [101, 102].

Olive or olive-derived products, particularly extra virgin olive oil, contains various phenolic compounds [103, 104]. Polyphenols in olives account for 1–2% of the fresh weight of the fruits, of which the most abundant classes of polyphenols include phenolic acids, phenolic alcohols, flavonoids, and secoiridoids [105]. The components and abundance of phenolic compounds in olive change during olive maturation. For example, oleuropein, a predominant secoiridoid, is a bitter taste compound in the olive, and the concentration of oleuropein is accumulated during the generation of fertilized pistils [106] and sharply reduced as it is hydrolyzed into hydroxytyrosol and elenolic acid glucoside during the olive maturation process [107].

Most dietary polyphenols are in the form of esters, glycosides, or polymers, which are unlikely to be absorbed in the native form by the intestine. Flavonoids are the extensively studied polyphenols, and most flavonoids also non-covalently bind to a glucose in plants. Hydrolysis of flavonoid glycoside form begins in the mouth where flavonoids are partially released from the food matrix by saliva [108]. While flavonoids polymers are degraded into small units which result from the low pH in the stomach [109], most flavonoids are hydrolyzed and further metabolized in the small intestine [110, 111], in which intestinal β-glucosidases cleaves glucose from flavonoid glycoside form, generating the aglycone form [112]. Hydrophobic flavonoids pass the apical membrane via passive diffusion, while other flavonoids in glycosylation form can be transported into enterocytes via SGLT1 [113]. Afterwards, the absorbed flavonoids are conjugated with glucuronic acid by uridine-diphosphate-glucuronosyltransferase in enterocytes, depending on the structure of flavonoids. For instance, flavonoids containing a hydroxyl group on the B-ring is less

predisposed to glucuronidation, but flavonoids containing catechol is prone to glucuronidation [114]. Flavonoids that are absorbed from the small intestine are passed to the liver where they can be further conjugated with sulfate and/or methyl groups and result in bile excretion [115-117]. The majority of ingested flavonoids may not undergo hydrolysis or conjugation in the small intestine [118], and are not absorbed [117]. Instead, these unabsorbed flavonoids reach the colon where they are degraded by colonic bacteria into smaller molecules and phenolic acids, which can then be partially absorbed into the circulation [119, 120]. Specifically, some intestinal bacteria strains have enzymatic activity of glucuronidase and glucosidase that deconjugate the glucuronidation or glycosylation form of flavonoids, and yield simple deconjugated products that are absorbed and metabolized by colonic enterocytes.

### *3.1 Polyphenols and GLP-1 secretion*

Some polyphenols or polyphenol-enriched extracts reportedly induce GLP-1 secretion. For example, oral oleuropein (20 mg) administration greatly increased postprandial GLP-1 concentrations in healthy people [121]. Consistently, oral administration of an olive oil-enriched diet increased plasma concentrations of GLP-1, and postprandial GLP-1 secretion in healthy rats [122]. However, it is unclear the major component of olive oil responsible for the observed effect on GLP-1, which could be MUFAs because polyphenol contents in oil olive is typically low [105]. Another study showed that extra virgin olive oil-enriched meal (containing oleuropein and other phenolic compounds) greatly elevated circulating GLP-1 concentrations with a 2.2-fold increase at 1 h after meal intake, and 4.7-fold at 2 h after meal intake [123]. In addition to the olive-derived polyphenols, several other polyphenols have been reported to induce GLP-1 secretion. For example, the derivatives of caffeoylquinic acid (CQA), the phenylpropanoids, elevated GLP-1

secretion in cultured clonal L-cells [124]. In vivo, a single intragastric administration of CQA-enriched diet potentiated the GSGS in rats [124]. Consistently, CQA-enriched coffee consumption significantly increases plasma GLP-1 levels in rodents and humans [125, 126]. As aforementioned, GLP-1 secretion from L-cells is triggered by the increased intracellular ATP level, which sequentially leads to the closure of the ATP-dependent $K^+$ channel, membrane depolarization, $Ca^{2+}$ influx, and ultimate GLP-1 secretion. While the underlying mechanism of CQA-induced GLP-1 secretion is still unclear, it was shown that CQA and its derivatives increased intracellular ATP production in clonal neuronal cells [127]. Curcumin, a polyphenolic turmeric, has been found to induce GLP-1 secretion from GluTag L-cells and improved GSGS in rats [128, 129], which might be mediated via GPR40/120, a fatty acid receptor [129]. In addition to curcumin, delphinidin 3-rutinoside (D3R), one of the anthocyanin derivatives from berry, was found to induce GLP-1 secretion from L-cell line via the GPR40/120-mediated mechanism [130]. Consistently, oral administration of anthocyanins-enriched blackcurrant extracts significantly elevated GSGS in rats [131]. Resveratrol, a polyphenol from grape seeds and skin, elevated plasma GLP-1 concentrations in mice through feeding a high-fat-diet for 5-weeks [132]. A recent study using computerized modeling predicted that resveratrol is an effective inhibitor of human DPP-IV through forming hydrogen bonds between catalytic residues of DPP-IV and hydroxyl group of resveratrol. In fact, it was demonstrated that resveratrol is a potent DDP-IV inhibitor with the $IC_{50}$ of 0.6 nM in a cell free assay system using porcine kidney DPP-IV [133]. However, unlike DPP-IV inhibitor drug [134], oral administration of resveratrol did not improve GLP-1 concentrations or glycemic control either in diabetic or obese patients [135, 136]. Therefore, those results from *in vitro* DPP-IV assays or mouse models likely lack the physiological relevance to humans.

### *3.2 The role of microbiota in polyphenol-stimulated GLP-1 secretion*

In addition to directly targeting L-cells to regulate GLP-1 secretion, polyphenols might modulate GLP-1 secretion in an indirect manner. In that regard, microbiota might be involved in bridging ingested polyphenols to the release of GLP-1 from L-cells. The human gut hosts a vast diversity of microorganisms that have been the subject of extensive research in recent years, as they may play important roles in human physiology, metabolism, and disease. Before weaning, the composition of intestinal microbiota is simple, relatively unstable, and undergoes drastic changes [137], while the composition of gut microbiota in healthy adults is substantially stable over time [138, 139]. However, differences in dietary composition have been shown to profoundly influence gut microbiota composition [140-142]. As to nutrient metabolism, gut microbiota primarily metabolizes non-digestible nutrients into SCFAs and gas. The change of diet affects the production of gut microbiota products, as the abundance of SCFA-producing bacteria is modified by the shift in dietary pattern. Resistant starch, a form of fiber that escapes intestinal digestion, elevates the relative proportion of *Eubacterium hallii*, which is the key species for butyrate production [140], and thereby increases the content of butyrate in the feces [143].

Recent studies have shown that there is a reciprocal relationship between microbiota and some polyphenolic compounds. First, non-absorbed polyphenols were fermented by various colonic microbiota based on their chemical structure to generate various metabolites [144]. For example, resveratrol was fermented by *Slackia equolifaciens* and *Adlercreutzia equolifaciens* to produce lunularin. Second, polyphenols modulated the population of the microbial community. Some polyphenols have antibiotic activity and can suppress the growth of pathological bacteria when cocultured with fecal microbiota. For instance, when intestinal bacteria were cultured with polyphenolic compounds from tea extracts, the growth of pathological bacteria, *Clostridium*

*perfringens*, *Clostridium difficile*, and *Bacteroides spp* was inhibited, but the abundance of the probiotic bacteria was not affected [145]. In addition, polyphenols exhibit a prebiotic-like effect, as they can reverse dysbosis induced by feeding a high-fat-diet. For example, the polyphenols that were extracted from concord grapes and fermented green tea were found to reduce the ratio of Firmicutes/Bacteroidetes, which was elevated in rodents fed a high-fat-diet [146, 147]. It was also found that the grape polyphenols restored the composition of microbiota via increasing the growth of *Akkermansia muciniphila* in the mice fed the high-fat-diet, which is an important bacterium for degrading mucin [146]. Further, as polyphenols modulate the microbial abundance, they might also alter the relative production of microbial metabolites. SCFAs and gas ($H_2S$) are the main microbiota metabolites of non-digestible food components. As aforementioned, SCFAs, primarily acetate, propionate, and butyrate, can trigger GLP-1 secretion *in vitro* and *in vivo* via their GPCRs [61, 79]. It was shown that $H_2S$ enhanced GLP-1 release both *in vitro* and *in vivo* [148]. *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *E. hallii* from Firmicute phyla are the most abundant bacteria in human feces, which have a major role in butyrate production [149]. *F. prausnitzii* functions as butyryl coenzyme A (CoA):acetate CoA transferase and acetate kinase, and it converts two molecules of acetyl-CoA to butyrate [150]. *E. rectale* and *E. hallii* catalyze butyrate formation from lactate and acetate [151]. A study reported that coculture of human fecal bacteria with 150 mg/ml and 1000 mg/ml tea catechins enhanced the growth of *E. rectale* [152]. Exposure of fecal microbiota to phenolic extracts from cocoa increased the production of SCFAs, whereas there were no significant changes in the proportion of butyrate-producing bacteria [153], suggesting that phenolic extracts from cocoa might modulate the activity of enzymes that associated with these bacteria. As *E. rectale* plays a vital role in butyrate production, elevated *E. rectale* population could lead to increased butyrate generation, which might ultimately induce

GLP-1 secretion. Oral administration of trans-resveratrol and quercetin reversed the decreased proportion of butyrate-producing bacteria that was induced by obesity [154], whereas the treatment failed to enhance SCFAs production. While studies in this area are still very limited, these available studies provide evidence that dietary polyphenolic compounds may play health beneficial roles via inducing GLP-1 secretion and promoting beneficial gut bacteria function.

### *3.3 The role of polyphenols in gut hormones-related food intake regulation*

Polyphenols or polyphenol-enriched diets have been reported to suppress food intake [155, 156]. Therefore, dietary incorporation of these natural compounds may potentially help with body weight control. Human appetite is largely regulated by central nervous system, particularly the hypothalamus [157]. The arcuate nucleus (ARC) in the hypothalamus primarily regulates food intake through regulating the expression of neurotransmitters or neuromodulators. ARC of hypothalamus is a group of neurons which contains the orexigenic center and anorexigenic center. Agouti-related peptide (AgRP) and neuropeptide Y (NPY) are the main neuropeptides in the orexigenic center. Proopiomelanocortin (POMC) is a precursor of polyneuropeptides in the anorexigenic center of hypothalamus. As ARC of hypothalamus is not fully protected by the blood-brain barrier (BBB) [158], several gut hormones, such as GLP-1, ghrelin, peptide YY (PYY), and cholecystokinin (CCK), have been reported to regulate appetite via modulating hypothalamic neuropeptide expression [159-162]. Indeed, long-term administration of GLP-1 leads to reduced food intake and moderate body weight loss in obese individuals [163], confirming that it plays a role in regulating body weight. Given polyphenols induce GLP-1 secretion *in vivo*, it is convinced that polyphenols affect appetite via modulating the release of the hormones or hormone action involved in the regulation of food intake. In addition, as ARC is not protected by BBB, it is possible

that the circulating polyphenols directly targets ARC to regulate neuropeptide expression, even though bioavailability of polyphenols in brain is extremely low [164]. Indeed, it was shown that oral administration of polyphenolic extracts from *Hibiscus sabdariffa* and *Lippia citriodora* reduced the feeling of hunger and increased the feeling of satiety through 2-month intervention in overweight or obese individuals, in comparison with placebo group, which was associated with an increased plasma anorexigenic hormone, GLP-1, and decreased orexigenic hormones, ghrelin, in experimental group [165]. Consistently, administration of polyphenolic extracts from *Hibiscus sabdariffa* and *Lippia citriodora* reduced body weight, body mass index (BMI), and fat mass in experimental groups [165]. Thus, the inhibitory effect on food intake and body weight results from the secretion of gut hormones that is induced by the administration of *Hibiscus sabdariffa* and *Lippia citriodora*. In addition, celastrol, a polyphenol isolated from *Tripterygium Wilfordi,* has been found to potently reduce food intake and promote body weight loss in diet-induced obese mice. Interestingly, the satiety and anti-obesity effect of celastrol was mostly due to its leptin sensitizing action [166]. While studies on the effects of polyphenols on neuropeptides are illustrative for understanding the polyphenols' role on food intake, the related studies are limited. A single dose of resveratrol (100 mg/kg) administration via intraperitoneal injection effectively suppressed food intake during 48 h after resveratrol administration in mice [155]. This study also demonstrated that resveratrol suppressed NPY and AgRP-driven luciferase activity in mouse hypothalamic cell line, which might be explained the reduced food intake with resveratrol administration *in vivo*. Furthermore, oral administration of proanthocyanidins-enriched grape extract (25 mg/kg) for 13 weeks reduced food intake and increased hypothalamic POMC mRNA abundance but not alter the body weight in obese rats [167]. However, a single dose of 500 mg grape extract administration has no effects on food intake or postprandial appetite response in non-

obese individuals [168]. The dose of proanthocyanidins-enriched grape extract (25 mg/kg) that used in the rat study is equivalent to 280 mg in the person with 70 kg body weight [169], which is lower than the dose of grape extract (500 mg) that used in the human study. In this regard, the inhibitory effect on food intake by oral grape polyphenols administration might be an accumulative action *in vivo*.

Results from investigating the effects of phenolic compounds on body weight control are not all consistent, which may be due to the different doses, age, and treatment duration. Polyphenols might have the potential to control appetite via modulating orexigenic or anorexigenic hormones, whereas it is unclear whether the reduced food intake by some tested phenolic compounds is due to the modulation of the plasma concentrations of orexigenic or anorexigenic hormones. Indeed, several studies showed that polyphenols administration altered body weight and fat mass, whereas food intake was not altered [170, 171]. It is well studied that dietary polyphenols inhibited the digestive enzymes in carbohydrate, protein, and fat digestion [172, 173]. For example, tea polyphenols suppressed α-amylase, pepsin, trypsin and lipase activity [174], as result of the non-covalent interactions between the polyphenols and enzymes [175]. And thus, the maldigestion that induced by polyphenols might lead to the reduction of body weight. In addition to regulation of digestion, several dietary polyphenols increased the energy expenditure in obese rodents and humans [176, 177], which might lead to the reduction of body weight. In this regard, the regulatory effect of polyphenols on body weight might be mediated by the comprehensive actions of polyphenols, accompanied by the alteration of food intake, digestive enzyme activity, and energy expenditure.

## 4 Conclusions

Loss of functional β-cell mass plays a central role for deterioration of blood glucose control in T2D. The GLP-1 signaling pathway has been extensively explored for developing the incretin-based drugs, as its activation can promote GSIS and β-cell mass. Polyphenols are a group of plant-derived compounds that may exert beneficial effect for human health. Emerging evidence shows that some phenolic compounds are capable of inducing GLP-1 secretion, and thus may be helpful in improving glucose homeostasis. However, this is still largely an unexplored territory and there remains many interesting questions that need to be addressed. It is unclear which specific category of phenolic compounds in the diet is superior than others in stimulating GLP-1 secretion. In addition, studies should be carried out to compare the efficacy of different categories of compounds, which may provide better understanding of the chemical structure-function relationship, and thus facilitate dissecting how a particular polyphenolic compound activates GLP-1 secretion from L-cells. Given most of the non-absorbed polyphenols were fermented by gut microbiota and some products derived from fermentation was found to induce GLP-1 secretion, it is of a vital importance to investigate how microbiota and which specific strain(s) involve in the regulation of GLP-1 secretion.

## 5 Figures

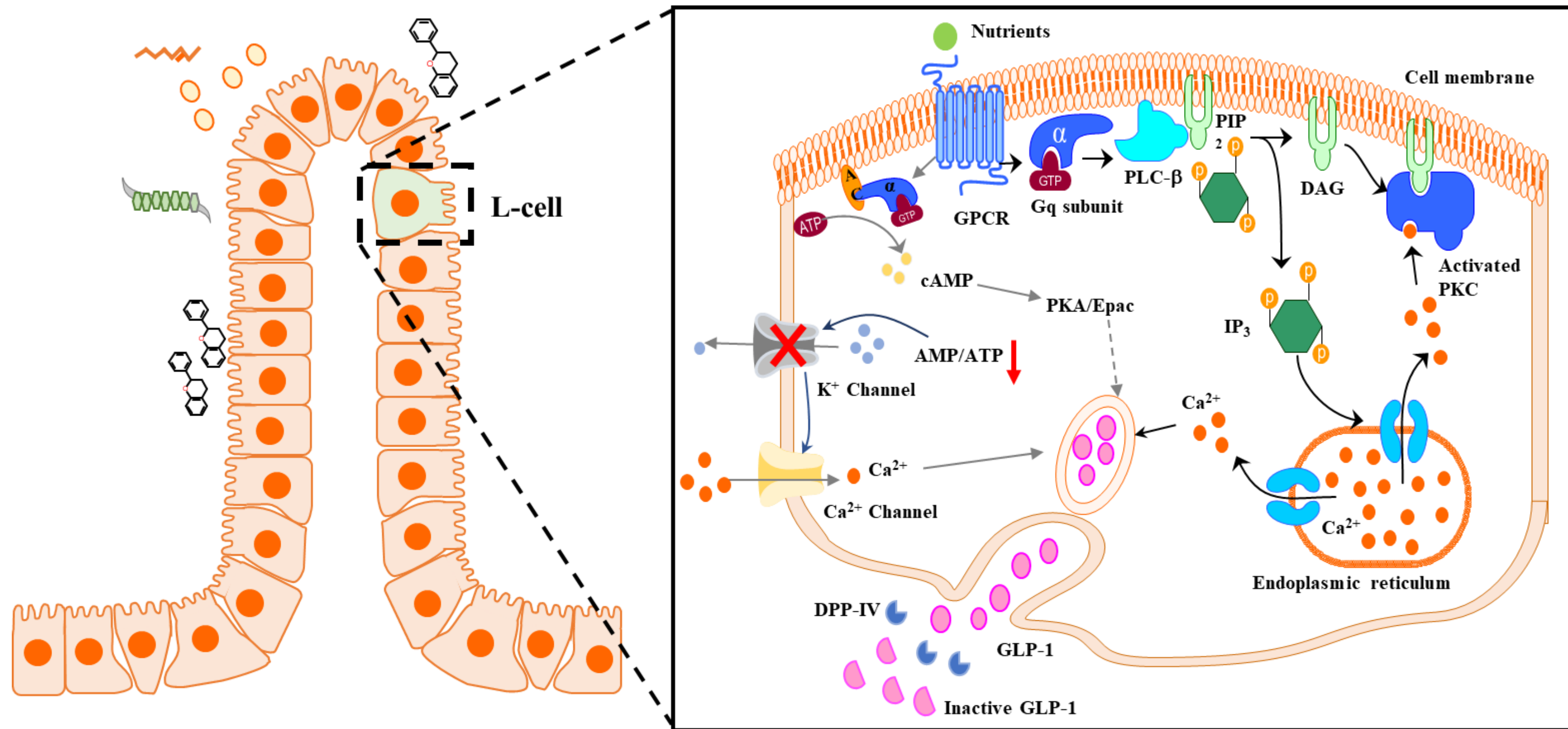


**Figure 1. Nutrients induce GLP-1 secretion from L-cell.**

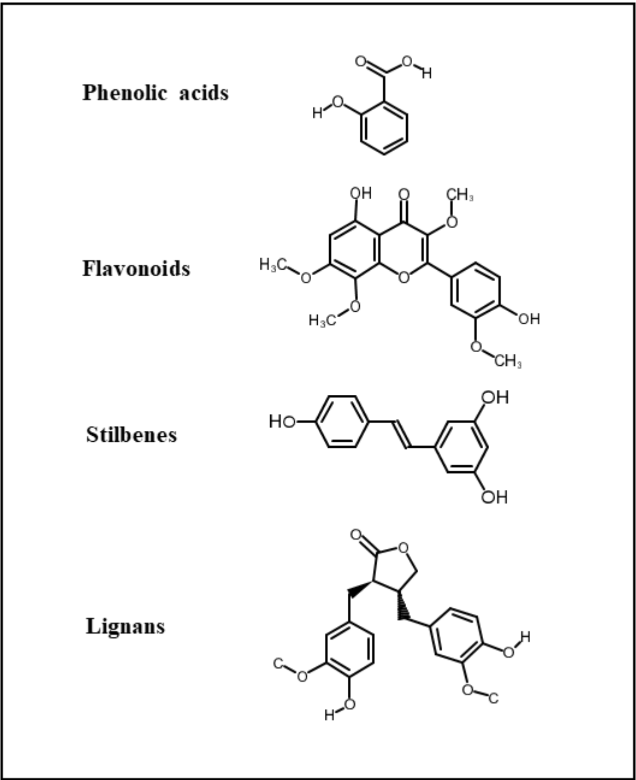


**Figure 2. Chemical structures of polyphenols from four categories**

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# CHAPTER THREE

## Small Molecule Elenolic Acid Is a Novel Agonist for GLP-1 Secretion

**Abstract**

Insulin resistance and β-cell dysfunction are the hallmarks of type 2 diabetes (T2D). The incretin hormone glucagon-like peptide-1 (GLP-1), which is secreted from intestinal L-cells, plays a critical role in maintaining glycemic homeostasis via potentiating glucose-stimulated insulin secretion (GSIS) and promoting β-cell proliferation and survival. Therefore, targeting L-cells for promoting its secretory function could be an effective strategy for treating and preventing T2D. Here, I report that elenolic acid, a small molecular compound present in mature olive, is a potent agonist for GLP-1 secretion *in vitro*. EA dose dependently stimulated GLP-1 release in both clonal L-cells and isolated mouse ileum crypts, with 10 μM inducing maximal effect. EA induced a rapid increase in intracellular $[Ca^{2+}]_i$ and the production of inositol trisphosphate in L-cells, indicating that EA activates phospholipase C (PLC)-mediated signaling. Inhibition of (PLC) ablated EA-stimulated increase of $[Ca^{2+}]_i$ and GLP-1 secretion in L-cells. In addition, EA-triggered GLP-1 secretion from L-cells was blocked by YM-254890, a $G\alpha_q$ inhibitor. Moreover, chronic exposure of GluTag cells to EA enhanced GLP-1 content and expression of proglucagon gene. These data provide evidence for the first time that EA might be a novel anti-diabetic agent through promoting endogenous GLP-1 secretion.

**Key words,** Elenolic acid, GLP-1, and L-cells

## Introduction

Type 2 diabetes (T2D) accounts for over 90% of diabetes in the United States [210]. Loss of β-cell mass and function is central to the development of T2D [211]. The incretin hormone glucagon-like peptide-1 (GLP-1), which is secreted from intestinal L-cells, plays a critical role in maintaining glycemic homeostasis via potentiating glucose-stimulated insulin secretion (GSIS) and promoting β-cell proliferation and survival [212, 213]. In addition, GLP-1 delays gastric emptying, promotes satiety, and reduces body weight in animal models of obesity [73]. Secretion of GLP-1 from intestinal L-cells is increased in response to ingested macronutrients, primarily fatty acids [14-16], even though glucose [97, 98], some amino acids [129], and dietary fibers [19] may also trigger GLP-1 release. In addition, a variety of neurotransmitters and neuropeptides released by the enteric nervous system and enteroendocrine cell types, such as acetylcholine [20] and gastrin-releasing peptide [22], have been implicated in the regulation of GLP-1 secretion. However, no therapeutic strategy based on these stimuli has been successfully developed for treating T2D.

There is accumulating evidence showing that dietary intake of olive (*Olea europaea* L.) olive oil or olive extracts provides metabolic benefits in humans with T2D [214, 215]. One recent study shows that oral administration of 500 mg olive leaf extract for 14-week decreased Hb1Ac and fasting plasma insulin concentration as compared to those in the placebo group of the patients with T2D [216]. Olive oil consumption has been found to reduce Hb1Ac and fasting plasma glucose concentration in patients with T2D [26]. In addition, daily intake of 15-20g olive oil attenuated the risk of T2D by 16% in humans [26]. However, the mechanism underlying the protecting effects of olive extracts or olive oil has not been well studied. The main components of olive or olive oil are lipids, sugars, and phenolic compounds [217]. Olive oil is rich in

monounsaturated fatty acids (MUFA) [30], which may play a role in health benefit of olive oil. Indeed, it was shown that MUFAs from an olive-oil containing Mediterranean diet improved glycemic control in the patients with insulin resistant [31]. However, there is no evidence showing that MUFA is the main contributor to the beneficial effect of olive in people with T2D [218].

Other than MUFA, extra virgin olive oil (EVOO) contains over 30 phenolic compounds [219]. Total polyphenols content in EVOO is 435 mg/L of gallic acid equivalents [27]. Previous studies have shown that consumption of EVOO, which has nearly identical fatty acid composition but considerably higher amount of phenolic compounds as compared with pure olive oil [220], improves glycemic control in both human subjects [221, 222] and animals [223]. Based on this finding, it is therefore presumed that the beneficial effect of EVOO may be primarily attributed to one or more of its phenolic components [223-225]. Oleuropein, ligstroside, oleuropein aglycone, hydroxytyrosol, and tyrosol are the main phenolic components in olive, olive oil or olive leaf. Several phenolic components that extracted from olive or olive oil improved glycemic control in rodents. It was shown that oral administration of tyrosol (20 mg/kg) attenuated fasting hyperglycemia in streptozotocin-induced insulin deficient rats, which was associated with enhanced activity of hexokinase and glucose-6-phosphate dehydrogenase [28]. In addition, oral administration of oleuropein-enriched diet alleviated hyperglycemia and improved oral glucose tolerance in obese diabetic mice [29]. However, the specific targets for these phenolic compounds to exert the observed effects are unclear, given their poor bioavailability from dietary intake.

Interestingly, a previous study showed that oral administration of an olive oil-enriched diet increased plasma concentrations of GLP-1, and postprandial GLP-1 secretion in healthy rats [156]. However, another study showed that EVOO-enriched meal greatly elevated circulating GLP-1 concentrations with a 2.2-fold increased at 1h after meal intake, and 4.7-fold at 2 h after meal

intake [27], suggesting that phytochemicals, rather than fatty acids in olive oil may largely be responsible for the observed effect on elevated circulating GLP-1. While it has been established that MUFA stimulates GLP-1 secretion *in vitro* and *in vivo* [31, 226], studies about the stimulatory effects of phenolic components on GLP-1 secretion are scarce. The composition of phenolic compounds in olive changes during fruit development and maturation. Specifically, for ripened olive, a bitter-taste compound, oleuropein, is hydrolyzed into elenolic acid (EA) and hydroxytyrosol [34]. Therefore, EA is considered a marker of olive maturation. EA is a small molecule with molecule weight of 242 g/mol, and it is one of derivatives of phenolic components from EVOO, with an average content of 10.4 mg/kg [35]. However, it has never been investigated, as to the best of our knowledge, whether it possesses anti-diabetic potential. Here, I investigated the effects of EA on GLP-1 secretion by using mouse GLP-1-secreting clonal L-cell line (GluTag) and further elucidated the underlying mechanism of EA-induced-GLP-1 secretion.

## Materials and Methods

### Chemicals

Oleuropein was from Shaanxi Huike Botanical Development Co. (Xian, China); U73122 was from Tocris Bioscience (Pittsburgh, PA);Vildagliptin was from Cayman (Ann Arbor, MI); YM 254,890 was ordered from Focus Biomolecules (Plymouth Meeting, PA); DMEM media, fetal bovine serum (FBS), and other cell culture supplements were obtained from Hyclone (GE Healthcare Bio-Sciences, Pittsburgh, PA); Fluro-4AM was from ThermoFisher Scientific (Waltham, MA); High-Capacity cDNA Reverse Transcription Kit and Power SYBR™ Green PCR Master Mix were from Applied Biosystems (Grand Island, NY); GLP-1 ELISA kit was from MilliporeSigma (Burlington, MA); antibody to mouse $G\alpha_q$ was from Santa Cruz Biotechnology (Dallas, Texas); $IP_3$ ELISA kit was from Amsbio (Cambridge, MA). All the other chemicals mentioned in this Chapter were from Sigma (St. Louis, MO).

### Generation of EA

The generation of EA was performed by the laboratory of our collaborator (Dr. Bin Xu). Briefly, oleuropein (purity ≥80%, HPLC) was dissolved in a versatile organic solvent, tetrahydrofuran (THF), followed by the addition of 1 N aqueous $H_2SO_4$ and stirring for 48 h at room temperature. After removing THF under vacuum laboratory, the solution was neutralized to pH 7.5 with 1 N NaOH solution. The solution was washed with Ethyl acetate (EtOAc) for 3 times. The neutral solution was acidified with 1 N HCl and extracted with EtOAc. The organic layer was collected and dried with anhydrous sodium sulfate overnight. After evaporating the solvent, the residue was purified by flash chromatographic procedures, using dichloromethane-methanol (contain 1% acetic acid) as mobile phase to obtain oil-like EA. Multiple validation assays of EA

were performed, including liquid chromatography-mass spectrometry (LC-MS) for purity analysis and MS-MS for identification analysis, and $^{1}$H-NMR and $^{13}$C-NMR spectroscopic analysis. The estimated yield of EA was 25-35%.

**Cell culture**

GluTag L-cells were cultured as previous described [101]. Briefly, Cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5.5 mM glucose, 10% fetal bovine serum (FBS), and 1% penicillin streptomycin. When cells reached 80% confluence, they were trypsinized with 0.05% trypsin-EDTA and seeded into plates according to the experimental design. The passage number of GluTag L-cells in all the experiments was between 24 and 35.

**GLP-1 secretion measurement**

GluTag L-cells were seeded into 24-well plates pre-coated with poly-D-lysine. EA was dissolved in dimethylsufoxide (DMSO) with a stock concentration of 0.1 M. Before the treatment, cells were incubated with KRB buffer containing 0.2% BSA for 30 min and then treated with EA (0.1 μM, 1 μM, 10 μM, and 50 μM) or 1 μM forskolin (Fos) and 1 μM 3-isobutyl-1-methylxanthine (IBMX) for 1 h. For some experiments, cells were pretreated with various inhibitors for 30 min prior to the addition of 10 μM EA for another 1 h. Supernatants were collected for measuring GLP-1 using an assay kit (Millipore), which was then normalized to the protein content in the same sample.

The isolation of ileum crypts was performed as previously reported [227]. Briefly, 8 to 16-wk-old C57BL6/J male mice were euthanized by $CO_2$, followed by cervical dislocation. The ileum

was dissected and removed from the abdominal cavity and flushed thoroughly with pre-chilled Hanks Balanced Salt Solution (HBSS) containing 1 mM $CaCl_2$ and $MgCl_2$. After removal of the remaining adipose tissues and muscles, ileum was minced into 1-2 $mm^2$ pieces and then digested with 0.3 mg/ml collagenase XI solution at 37ºC and centrifuged at 100 g for 3 min. The collected ileum crypts were resuspended in DMEM supplemented with 25 mM glucose, 10% FBS, and 1% penicillin and streptomycin, and plated on Matrigel-coated 24-well plates. The crypts were maintained for 48 h, and then treated with various concentrations of EA or 10 µM forskolin plus 10 µM IBMX for 1 h to measure GLP-1 secretion.

**DPP-IV activity measurement**

The effect of EA on DDP-IV activity was assessed as previously reported [228]. Briefly, 2 mU/mL human DPP-IV was prepared in 100 mM Tris buffer (pH 8.0). EA was dissolved in DMSO and further diluted to 0-400 µM with 100 mM Tris buffer (pH 8). DMSO concentration of each dilution was normalized to 0.4%. In a 96-well plate, 50 µL DPP-IV buffer and 50 µL EA were mixed and incubated at 37ºC for 10 min followed by addition of 100 µL 0.5 mM GPpNA chromogenic substrate in 100 mM Tris buffer (pH 8.0). The plate was further incubated at 37ºC. After 1 h, the reaction was terminated with the addition of 50 µL acetic acid (3%), and the plate was read at the absorbance of 405nm. For blank measurement, 50 µL 100 mM Tris buffer was used instead of DPP-IV solution in the reaction. The DPPV-IV inhibitor, vildagliptin (10 µM) which was dissolved in the same buffer, was used as the positive control.

**Intracellular cyclic adenosine monophosphate (cAMP) measurement**

Confluent cells were washed with pre-warmed PBS twice and incubated with various concentrations of EA or with 0.2 mM IBMX for 20 min. The treated cells were then washed with ice-cold PBS, followed by incubation with 0.1 M HCl for 20 min. The cAMP contents in cell lysates were measured using an EIA kit according to the manufacturer's manual.

**Intracellular Calcium Concentration ($[Ca^{2+}]_i$) Measurement**

GluTag cells were trypsinized and washed with KRB buffer for 3 times. Cells were loaded with 2 µM Fluo-4AM (ThermoFisher Scientific) in $Ca^{2+}$-free KRB buffer at 37ºC for 1h. The cells were then washed and resuspended in $Ca^{2+}$-free KRB buffer at 2 x$10^6$ cell/mL and transferred to opaque 96-well microplate. For intracellular calcium measurement, $Ca^{2+}$-free KRB buffer containing EA, KCl, or inhibitors was injected into culture plates. The Fluo-4AM fluorescence data were recorded at 495 nm excitation and 518 nm emission using a spectrofluorophotometer (FLUOstar OPTIMA, Cary, NC). Basal signals of Fluro-4AM- loaded cells were recorded for 10 sec. After the addition of compounds, The oscillation of $[Ca^{2+}]_i$ was continuously monitored for up to 240 sec.

**$IP_3$ production measurement**

GluTag L-cells were trypsinized and washed with warmed PBS for 3 times. Cells ($10^5$ cells per well) were then treated with vehicle or 1 µM EA for 30 sec, followed by addition of 10% perchloric acid to terminate the reaction. The solution was neutralized with 1.5 M KOH. The reaction mix was centrifuged for 15 min at 1500×g. Supernatants were collected for measuring $IP_3$ concentrations using a mouse $IP_3$ ELISA kit (Amsbio).

**Trypsin sensitivity assay**

GluTag L-cells were lyzed in 10 mM Tris-HCl (pH 7.4) buffer containing 5 mM EDTA, 10 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor (type II-S), and 5 μg/ml leupeptin, and plasma membranes were then isolated as previously described [229]. For trypsin sensitivity assay, 30 μg cell membranes were incubated in 25 mM HEPES (pH 7.5) buffer containing 1 mM EDTA, 20 mM β-mercaptoethanol, 25 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, and 50 μM GTPγS. in the presence or absence of EA or vehicle at 37ºC for 15 min. Afterward, the cell membranes were digested with 100 μg/ml of N-tosyl-L-phenylamine chloromethyl ketone (TPCK)-trypsin (1:25 ratio of trypsin to total protein) at room temperature for 15 min, and the resulting cleaved products were analyzed by immunoblotting with antibodies against Gαs, Gαi, and Gαq.

**GLP-1 content assays**

GluTag L-cells were treated with various concentrations of EA or forskolin plus IBMX (FI, 1 μM for each) for 18h. Cells were then washed and lyzed in 50 mM Tris buffer containing 1 M HCl, 1% trifluoroacetic acid, 5% HCOOH and 1% NaCl [92]. GLP-1 levels in cell lysates were measured as described above and normalized to the protein contents.

**Quantitative real-time PCR (qRT-PCR) analysis**

GluTag L-cells were treated with various concentrations of EA or forskolin and IBMX (FI, 1 μM for each) for 18h. Total RNA in cell lysates was then isolated using the TRI reagent, and 1 μg RNA from each sample was reverse-transcribed to cDNA using a high-capacity cDNA reverse

transcription kit. The primer pair of mouse proglucagon was used according to previous study [230]. Forward and reverse primer sequences of mouse Gapdh are 5'-TGTGTCCGTGGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTTGA-3'. The resultant cDNA was amplified using SYBR Master mix using 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative expression levels of proglucagon mRNA to Gapdh transcripts were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold of the control [231].

**Statistical analysis**

The data with multiple groups were analyzed by one-way ANOVA, followed by Duncan's multiple range test using JMP 13 (SAS Institute Inc, Cary, NC). For two group comparison, student t-test was performed. Values are presented as mean ± standard error of mean (SEM), and $p < 0.05$ was considered significant difference.

## Results

### Synthesis and validation of EA

EA is synthesized by hydrolytic reaction of oleuropein followed by multiple steps of extractions and purification procedures. There are two forms of EA, aldehyde form (also called dihydropyran form) and hemiacetal form, while the hemiacetal EA is not stable. The purity of generated EA was determined by LC-MS analysis, which showed that predominantly EA peak with retention time of 5.50 min [M-H]- of 241.07 (Fig. 1A). The $[M\text{-}H]^-$ ion and dimer ion $[2(M\text{-}H)+Na]^-$ of EA were the most predominant species and its identification was further confirmed with high-resolution mass spectrometry fragmentation (Fig. 1B). Excellent yield (25-35%) of EA was obtained in large gram-scale synthesis work. Inlet showed chemical structure of EA and calculated mass. The aldehyde form of EA was determined by Nuclear Magnetic Resonance (NMR) spectroscopy (Fig. 1C and D). The chemical shift of aldehyde EA is 13C (>200 ppm) and 1H (9.7 ppm) from $^{13}C$-NMR and $^{1}H$-NMR analysis, respectively.

### EA was an intestinal L-cell functional agonist

To test whether EA stimulates GLP-1 secretion, I treated GluTag L-cells, a mouse L-cell line, with EA (0.1, 1, 10, and 50 μM) for 1 h, and then measured GLP-1 secreted in the medium. The result showed that EA promoted GLP-1 secretion from L-cells, with 10 μM inducing significant GLP-1 release (Fig. 2A). Isolated mouse primary ileum crypts were used for determining whether EA stimulates GLP-1 secretion from primary L-cells. The primary ileum crypts were cultured and treated with or without EA. Similarly, EA treatment significantly increased GLP-1 secretion from ileum crypts (Fig. 2B), suggesting that EA effect on L-cells is physiologically relevant.

**EA was not a DPP-IV inhibitor**

First, GluTag L-cells were pre-incubated with 10 μM vildagliptin, a DPP-IV inhibitor, for determining whether EA induces GLP-1 secretion through inhibition of DPP-IV. As shown in Fig. 3A, vildagliptin alone increased GLP-1 secretion by 16% ($p < 0.05$), which was only about one-third of that achieved by exposure to EA. In the presence of vildagliptin, EA-stimulated GLP-1 release was further increased. In addition, vildagliptin was potent in inhibiting DPP-IV with 10 μM ablating its enzymatic activity by over 70%, whereas EA at the same doses was inactive (Fig. 3B). These results demonstrated that EA's action on GLP-1 secretion from L-cells was mediated through a DPP-IV-independent mechanism.

**EA induced-GLP-1 secretion was not cAMP-dependent**

GluTag L-cells were incubated with various concentrations of EA or with 0.2 mM IBMX for determining whether EA induces GLP-1 secretion via increasing intracellular cAMP. Cell lysates were collected for intracellular cAMP measurement. As shown in Fig. 3C, EA did not significantly affect intracellular cAMP content ($P = 0.26$ in 10 μM of EA), even though the cAMP content trended to increase in higher concentration of EA. However, IBMX increased cAMP concentration by 1.5-fold over the vehicle-treated cells, suggesting that EA-induced GLP-1 secretion is cAMP-independent.

**EA induced-GLP-1 release was dependent on PLC/$Ca^{2+}$signaling**

To explore the signaling mechanism that mediated EA-stimulated GLP-1 secretion, I first examined whether EA increased $[Ca^{2+}]_i$, which is critical for triggering GLP-1 secretion [232]. The result showed that L-cells exposed to EA displayed a rapid increase in $[Ca^{2+}]_i$, a magnitude comparable to that elicited by 50 mM KCl (Fig. 4A). Next, I examined the effect of EA on the activity of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to the $Ca^{2+}$-mobilizing second messenger inositol triphosphate ($IP_3$), thereby elevating cytosolic $[Ca^{2+}]$ [106]. The result showed that EA treatment elicited a rapid $IP_3$ production in L-cells, indicating that EA stimulated PLC activity (Fig. 4B). GluTag L-cells were pre-treated with U73122, a specific antagonist of PLC [233], followed by addition of EA for measuring GLP-1 secretion, for determining the role of PLC in EA-stimulated GLP-1 secretion. The blockage of PLC completely ablated EA-elicited $[Ca^{2+}]_i$ increase (Fig. 4C) and GLP-1 secretion (Fig. 4D) from L-cells, suggesting that EA induced-GLP-1 secretion was mediated via a PLC-dependent pathway.

**EA stimulated-GLP-1 secretion was mediated via $G\alpha_q$.**

The next aim was to determine whether EA induces GLP-1 secretion via $G\alpha_q$, as the secretion of GLP-1 could be regulated via G-protein-coupled receptors (GPCRs) [234], which signal primarily through $G\alpha_q$, leading to activation of $PLC/Ca^{2+}$ signaling [14, 15, 109-111]. In that regard, the cells were pre-incubated with the $G\alpha_q$ specific inhibitor, YM 254, 890, for 30 min, followed by addition of EA (10 µM) for 1 h. The blockage of $G\alpha_q$ diminished EA-induced GLP-1 secretion (Fig. 5A). Trypsin sensitivity assay was performed as described [229, 235] for examining whether EA activates $G\alpha_q$, which is based on the observation that the agonist-stimulated binding of GTP to the $G\alpha$ subunit of the G-protein protects it from digestion by trypsin [229]. To that end, the isolated plasma membranes from GluTag L-cells were treated with EA or vehicle for 15 min,

followed by immunoblot analysis of the cleaved proteins with antibody against $G\alpha_q$. Cell membrane incubated with EA exhibited a clear and strong $G\alpha_q$ band, while the cell membrane incubated with vehicle did not show a band (Fig.5B). The result demonstrated that EA-activated $G\alpha_q$ was protected from the cleavage of trypsin, further confirming that $G\alpha_q$ was required for EA-induced GLP-1 secretion from the cells.

**EA enhanced GLP-1 synthesis**

Our preliminary data showed that EA did not have any effect on GLP-1 production in 3h, 6h, and 12 h of the treatment (Data not shown). To determine whether treatment with EA modulates GLP-1 synthesis in a chronic manner, I incubated cells with various concentrations of EA or forskolin and IBMX for 18 h, and intracellular GLP-1 contents were then measured. The result showed that EA dose-dependently increased GLP-1 contents (Fig. 6A), with 10 μM exerting the maximal effect. As the GLP-1 is encoded by proglucagon gene, the increased GLP-1 synthesis is associated with the increased expression of proglucagon gene. To evaluate whether EA enhances the expression of proglucagon gene, I incubated GluTag cells with various concentrations of EA or forskolin/IBMX for 18 h and then isolated RNA for qPCR analysis. EA induced proglucagon gene expression, with 10 μM of EA induced a significant increase (1.5-fold) over the control (Fig. 6B). In addition, the pattern of increase in proglucagon gene was consistent with the pattern of increase in GLP-1 content, suggesting that the increased GLP-1 production might result from the increased proglucagon expression.

**Discussion**

Some naturally occurring compounds have been reported to exhibit anti-diabetic effects, which provide a wide source for new drug discovery. Given that GLP-1 plays a vital role in maintaining glucose homeostasis via potentiating GSIS and promoting pancreatic β-cell proliferation and survival, targeting GLP-1 has been a novel therapeutic avenue for treating T2D. Here, I found that, for the first time, EA, a small molecule component from olive and EVOO, stimulates GLP-1 secretion via activation of the $G\alpha_q$-PLC-$IP_3$-$Ca^{2+}$ pathway. Therefore, it is attempting to speculate that EA might exert anti-diabetic potential due to its stimulatory effect on GLP-1 secretion.

While nutrients stimulate GLP-1 secretion through different pathways, the primary mechanism that induces the GLP-1 granule exocytosis from intestinal L-cell is the increase of $[Ca^{2+}]_i$ [236], which could arise either by release from intracellular calcium stores or by the influx from the calcium channel on the cell membrane. Indeed, glucose stimulated GLP-1 secretion in the GluTag cells via a series of cellular and membrane current activity, including the closure of the ATP sensitive potassium ($K_{ATP}$) channel, and opening of the voltage calcium ($Ca_v$) channel, which results in the calcium influx into the cytosol and the increased $[Ca^{2+}]_i$ [237]. In addition, fatty acids-stimulated GLP-1 release primarily depends on G-protein coupled receptors (GPCRs) on the cell membrane [109], of which the activation of GPCRs leads to the $IP_3$-mediated $Ca^{2+}$ release of intracellular calcium store, as a result of the PLC action, and ultimately results in the increased $[Ca^{2+}]_i$. In present study, EA induced a transient and fast increase of $[Ca^{2+}]_i$ in the GluTag cell, which is consistent with the well-established mechanism that the stimulated GLP-1 secretion is $[Ca^{2+}]_i$ dependent.

It is well studied that $G\alpha_q$-activated pathways are involved in several hormones' secretion, in particular, insulin [238], GLP-1 [109], growth hormone [239], and parathyroid hormone [240]. $G\alpha_q$-activated GLP-1 secretion depends on specific receptors, based on the chemical structure of ligands. Interestingly, GluTag cells express a wide variety of $G\alpha_q$-coupled receptors, and most of them have been shown to be involved in the GLP-1 secretion, such as receptors of fatty acids [238], amino acids [125, 126], and neurotransmitters [20]. Here, EA stimulated GLP-1 secretion via the $G\alpha_q$-activated pathway in GluTag cells, suggesting that EA might bind with a $G\alpha_q$-coupled receptor to exert its stimulatory action. Indeed, short chain fatty acids (SCFAs) induce GLP-1 secretion via the activation of fatty acid receptors [15, 114], and thereby trigger the $G\alpha_q$-activated pathway [15, 241]. Given that the binding of SCFAs to the receptors might depends on the carboxylic acid group [242], the chemical structure of EA showed a carboxylic acid group on the benzene ring, which suggests that EA might be a ligand of GPCRs. In this regard, EA might activate the $G\alpha_q$-mediated signaling pathway via the specific membrane receptor.

In addition to the stimulatory action of EA on GLP-1 secretion, EA elevated the GLP-1 production and proglucagon gene expression in GluTag cells. The pattern of GLP-1 production and proglucagon gene expression in response to various concentrations of EA was similar, with the maximum effect at 10 μM. It is well studied that proglucagon gene expression is regulated by protein kinase A (PKA)-dependent manner in GluTag cells [243]. The activation of PKA depends on the binding of cAMP with its two regulatory subunits and thereby exerts signal transduction [244]. In this study, though there was no significantly stimulatory effect of EA on intracellular cAMP in GluTag cells, the cAMP trended to increase in higher concentration of EA, suggesting that the regulatory effect of EA on proglucagon gene expression might be mediated by cAMP pathway. However, other pathway might be also involved [245]. Indeed, intestinal proglucagon

gene expression is reported to be regulated by β-catenin of the Wnt signaling pathway [246], where the activation of β-catenin induces the increase of proglucagon gene expression and GLP-1 production. Another explanation for the inconsistency between increase of proglucagon gene expression and cAMP is the different treatment period in these two experiments. The cell was treated for 20 min for cAMP measurement, but 18 h for assessing proglucagon gene expression. If EA binds to the receptor on the cell membrane to induce the stimulation of GLP-1, the stimulatory action should be a fast and acute effect. However, EA induced-GLP-1 production and proglucagon gene expression is a chronic effect (18 h treatment), suggesting that the effect of EA may be cumulative.

While the safety of EA *in vivo* is largely unclear, EA is isolated from EVOO and mature olive, which were widely consumed by humans, suggesting that EA is conceivably safe. Results show that at least 10 μM EA is needed to achieve the statistically stimulatory effect on GLP-1 secretion *in vitro*. Thus, it is necessary to do the initial *in vivo* study to test stimulatory effect EA in mice and the most effective dose in the further animal studies.

In conclusion, I found that for the first time, to the best of our knowledge, a small molecule from mature olive and EVOO, EA effectively stimulated GLP-1 secretion *in vitro* via activation of the $G\alpha_q$-PLC-$IP_3$-$Ca^{2+}$ signaling pathway. Given that GLP-1 plays a vital role in maintaining glycemic homeostasis, increasing GLP-1 secretion could be a therapeutic avenue for treating T2D. As EA stimulated GLP-1 secretion from clonal L-cell line and primary ileum crypts, it is likely that EA has the anti-diabetic potential, however, further study is still needed to determine whether EA enhances GLP-1secretion *in vivo* and exerts anti-diabetic action.

**Figures**

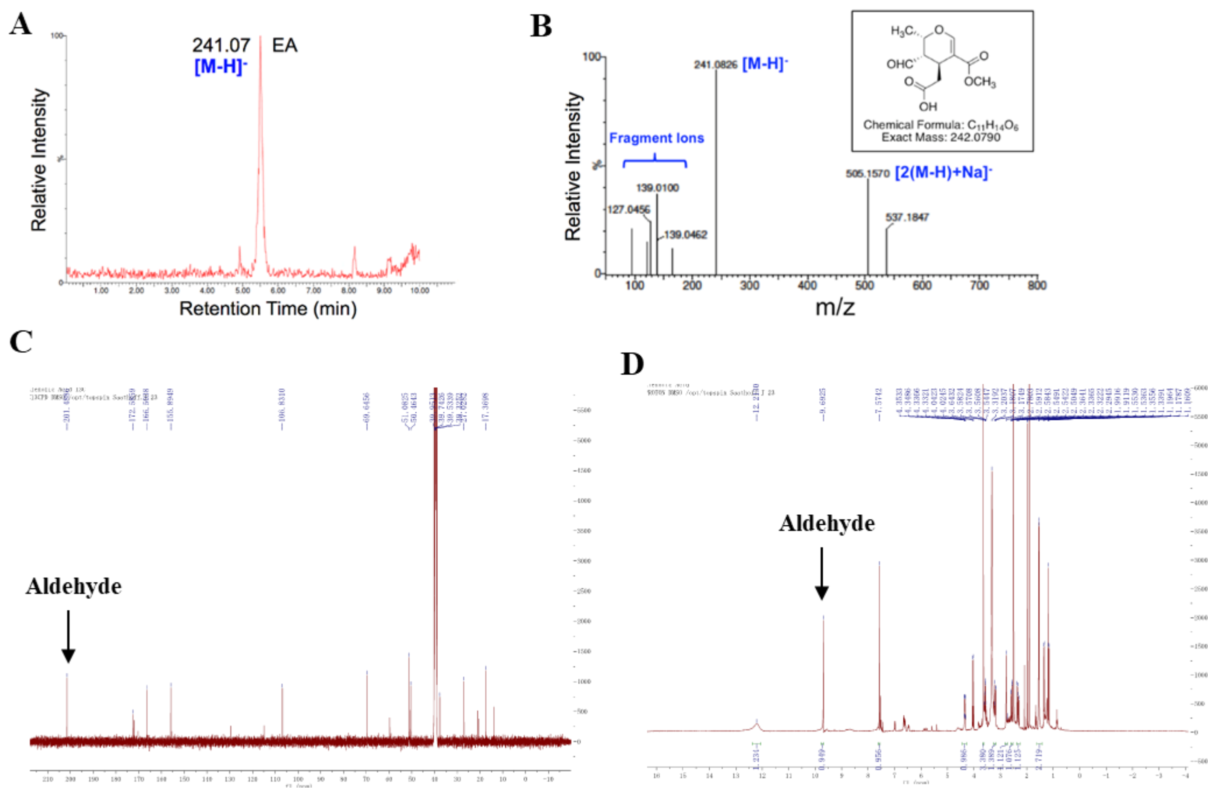


**Figure 1. Identity and purity analysis of purified elenolic acid (EA).** A. LC-MS analysis of purified EA. B. High-resolution mass spectrometry and validation of EA purified from oleuropein. Inlet shows chemical structure of EA and calculated mass. $^{13}$C NMR (C) and $^{1}$H NMR (D) spectra were performed to identify the aldehyde form of EA.

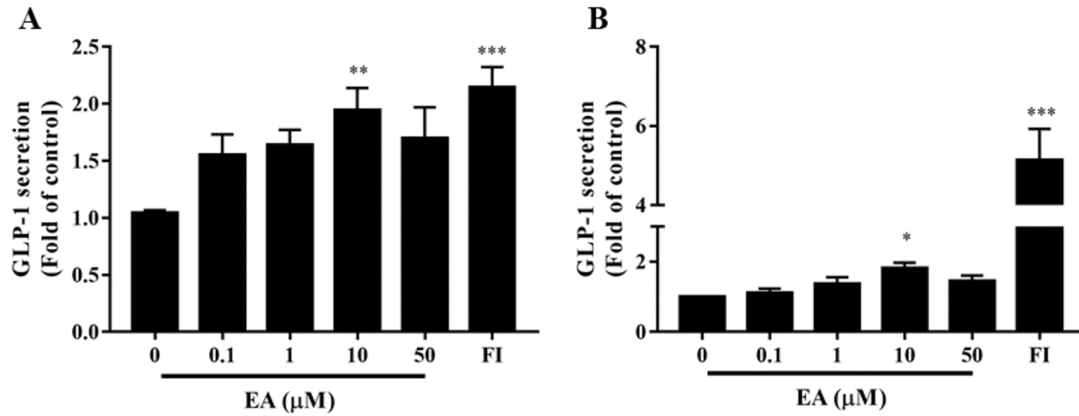


**Figure 2. EA dose-dependently stimulated GLP-1 secretion.** GluTag cell (A) and primary mouse ileum crypts (B) were treated with various concentrations of EA or forskolin and 3-isobutyl-1-methylxanthine (FI) (1 μM of each in GluTag cells and 10 μM of each in primary ileum crypts) for 1 h. Supernatants were collected for GLP-1 measurement. The GLP-1 secretion was normalized to protein content of each treatment. Data was presented as means ± SEM, n=5-6. * $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$ vs. control.

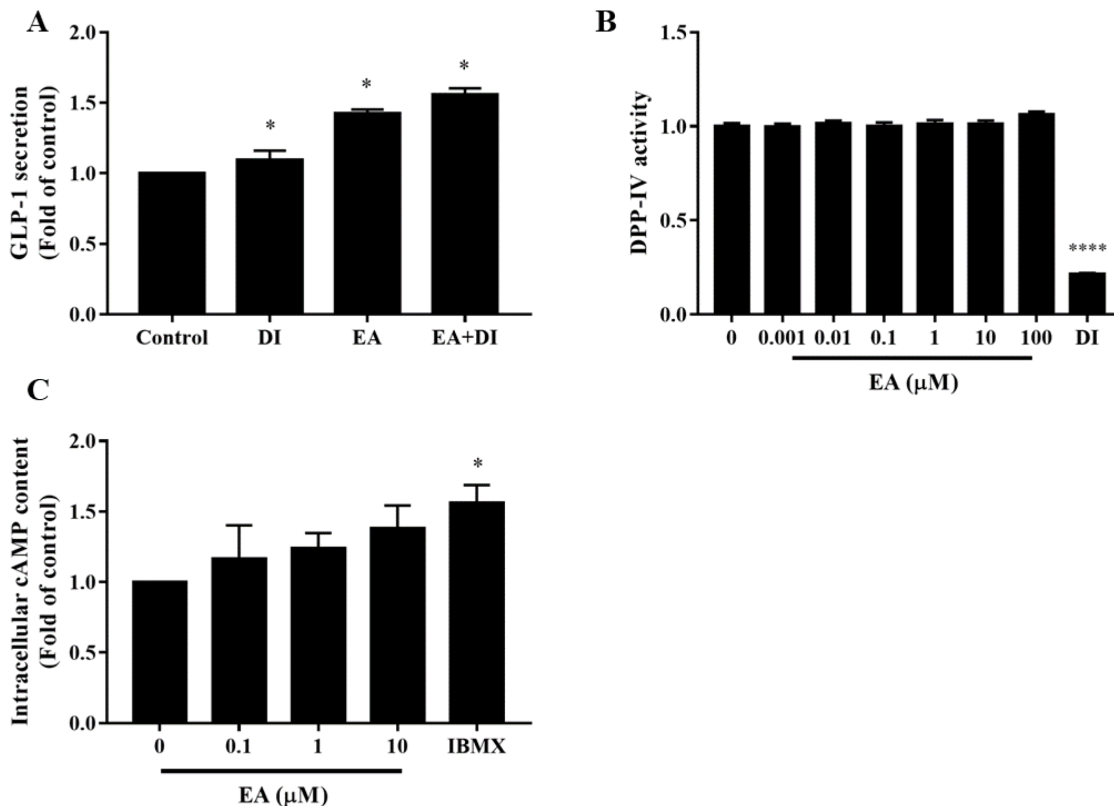


**Figure 3. EA has no effects on DPP-IV activity.** (A) GluTag cells were treated with 10 μM vildagliptin (DI), 10 μM EA, or DI and EA for 1h. GLP-1 secretion was measured and normalized with protein content of each treatment. (B) Human DPP-IV enzyme was incubated with various concentrations of EA or 10μM vildagliptin, DPP-IV inhibitor (DI) for 30 min. DPP-IV activity

was measured at the wavelength of 410 nm. (C) GluTag cells were treated with various concentrations of EA or 0.2 mM IBMX for 20 min. Cell lysates were used for intracellular cAMP measurement. Data was presented as means ± SEM, n=3. **** $p < 0.0001$ vs. control.

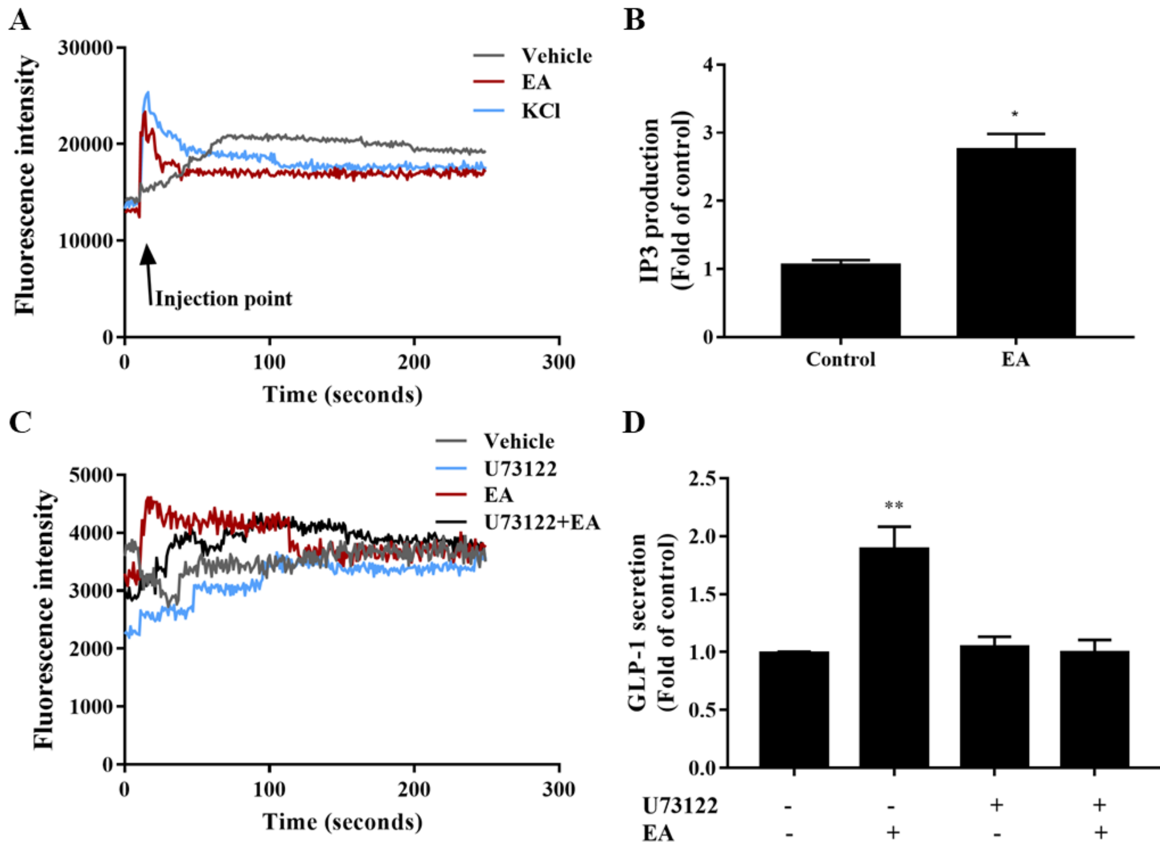


**Figure 4. EA induced-GLP-1 secretion is PLC-dependent.** (A) Suspended GluTag cells were pretreated with Fluo-4am and treated with vehicle, 10 µM EA, or 50 mM KCl. The $[Ca^{2+}]_i$ response was measured using fluorescence plate reader. The data was shown as the presentative of four independent experiments. (B) GluTag cells were pretreated with 10 µM U73122 for 30 min, and treated with vehicle or 10 µM EA for another 1 h. Supernatants were collected for GLP-1 measurement. (C) Suspended GluTag cells were stained with Fluo-4am and pretreated with 10µM U73122 for 30 min, and injected with vehicle or 10 µM EA. The $[Ca^{2+}]_i$ response was measured using fluorescence plate reader. The data was shown as the presentative of three independent experiments. (D) 10^6 cells were treated with vehicle and 10 µM EA. The intracellular $IP_3$ content was measured using ELISA kit. Data were presented as means ± SEM, n=4. * $p < 0.05$, ** $p<0.01$ vs. control

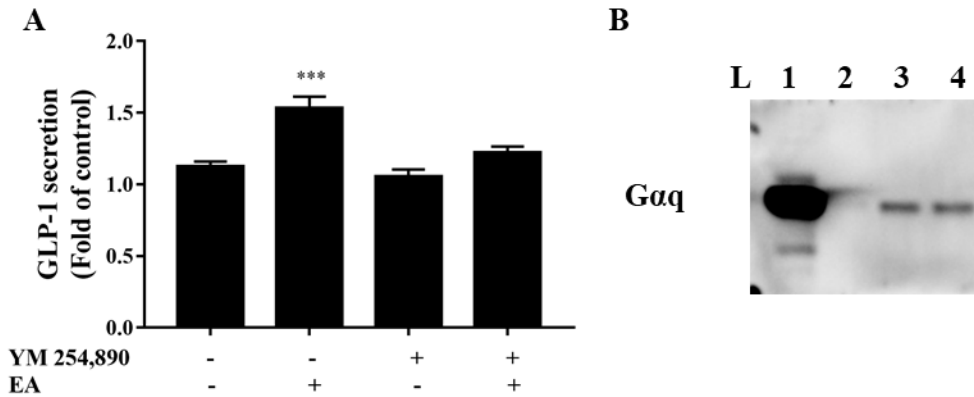


**Figure 5. EA-induced GLP-1 secretion is G$\alpha_q$ dependent.** (A) GluTag cells were pretreated with 10 µM Gαq inhibitor, YM 254,890 for 30 min, and treated with vehicle or 10 µM EA for another 1 h. Supernatants were collected for measuring GLP-1 secretion. (B) Trypsin sensitivity assay was performed to identify the specific subunit of Gα. Cell membranes were isolated from GluTag cells, and treated with vehicle, 1µM or 10µM EA for 15 min. Western blotting was performed with G$\alpha_q$ antibody. L: ladder; Lane 1: Assay control (without trypsin); Lane 2: Experiment control (vehicle); Lane 3: 1 µM EA; Lane 4: 10 µM EA.

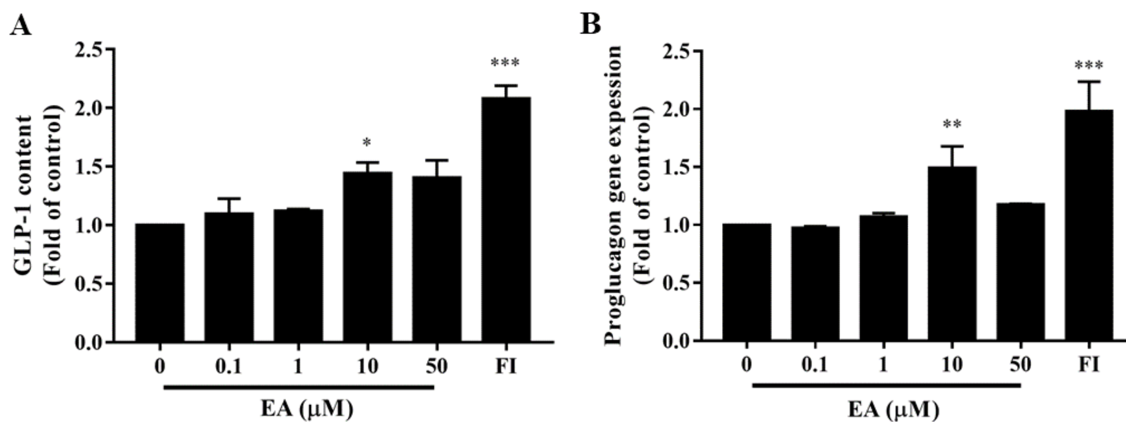


**Figure 6. EA increased GLP-1 content and gene expression.** (A) GluTag cells were treated with various concentrations of EA or 10 µM forskolin and 10 µM 3-isobutyl-1-methylxanthine (FI) for 18 h. Cell lysates were collected for GLP-1 content measurement. (B) GluTag cells were treated with various concentrations of EA or FI for 18 h. RNA was collected for qPCR analysis. Data were presented as means ± SEM, n=3. * $p < 0.05$, **$p<0.01$, ***$p<0.001$ vs. control.

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# CHAPTER FOUR

## Small Molecule Elenolic Acid Has Potent Anti-obesity and Anti-diabetic Activity in Diet-Induced Obese Mice

**Abstract**

Insulin resistance and pancreatic β-cell dysfunction are the characteristics of type 2 diabetes (T2D). Gut hormone, glucagon-like peptide 1 (GLP-1), plays a critical role in maintaining glycemic homeostasis via potentiating glucose-stimulated insulin secretion (GSIS) and promoting β-cell proliferation and survival, as well as improving insulin sensitivity. Agents that can directly promote GLP-1 secretory function of L-cells, thereby maintaining glucose homeostasis, holds great potential for the treatment of T2D. I found that olive-derived molecule, elenolic acid (EA) is a novel inducer of GLP-1 secretion from intestinal L-cells. In the present study, I further explored the anti-diabetic effects of EA using diet induced middle-aged obese (DIO) mice. Acute administration of EA stimulated GLP-1 secretion in mice with improved oral glucose tolerance. Once daily oral administration of 50 mg/kg EA for 7 weeks almost completely reversed hyperglycemia and glucose tolerance in DIO mice, which were associated with the improved insulin sensitivity. In addition, EA has strong anti-obesity effect, as it steadily promoted body weight loss and reversed obesity, which is primarily due to suppressed food intake. Consistently, EA delayed stomach emptying rate, increased anorexigenic peptide tyrosine tyrosine (PYY) secretion, and downregulated hypothalamic agouti-related protein (Agrp) expression. Further, EA reversed fatty liver and enhanced hepatic fatty acid oxidation. These results suggest that naturally occurring EA might be a novel anti-diabetic and anti-obesity compound.



**Key words**: Elenolic acid, GLP-1, food intake, diabetes, obesity, mice

## Introduction

Type 2 diabetes (T2D) is one of the fastest growing diseases in the United States where the incidence of T2D has doubled in the last five years [1]. T2D occurs more frequently in middle-aged adults. Indeed, patients with T2D between 45 and 64 years of age accounts for 46.5% of the patients with T2D in all ages [1]. Insulin resistance and pancreatic β-cell dysfunction are major determinants of T2D [2]. Obesity is a key risk factor for insulin resistance and diabetes as it impairs insulin-dependent glucose uptake in peripheral tissues, resulting in increased insulin secretion from pancreatic β-cells to overcome obesity-induced insulin resistance for glycemic control [247, 248]. Constant insulin resistance will progress to T2D when β-cells are unable to secret adequate amounts of insulin to compensate for decreased insulin sensitivity, which is largely due to pancreatic β-cell dysfunction and apoptosis [249-251]. Therefore, significant loss of functional β-cell mass over the time in patients with T2D plays a central role in the deterioration of blood glucose control [211, 249, 252, 253]. It has been well established that incretin hormones, which are released from enteroendocrine cells into the circulation in response to food ingestion, play an essential role in maintaining glucose homeostasis [254]. Indeed, incretin-stimulated insulin secretion accounts for over 50% of postprandial insulin secretion in healthy people [213, 255]. In particular, glucagon-like peptide 1 (GLP-1), a well-studied incretin secreted from intestinal L-cells [6], plays a vital role in maintaining glucose homeostasis via potentiating glucose-stimulated insulin secretion (GSIS) and promoting β-cell proliferation and survival [7-9]. In addition, GLP-1 suppresses appetite, delays gastric emptying and thus reduces food intake and controls body weight [76, 77, 256-258]. Ingested macronutrients, like fatty acids [14-16], proteins[17], and carbohydrates [18], as well as dietary fibers [19], induce GLP-1 secretion. As well, a variety of neurotransmitters and neuropeptides released by the enteric nervous system and enteroendocrine

cell types, such as acetylcholine [20] and gastrin-releasing peptide [22], have been implicated in the regulation of GLP-1 secretion. However, no therapeutic strategy based on these nutrients or hormonal factors has been successfully developed for treating T2D.

Olive or olive oil has drawn wide attention for health benefits in preventing or treating metabolic diseases such as T2D [26, 259], obesity [260, 261], and cardiovascular disease [262, 263]. A large cohort human study involving 185,671 individuals from Europe and United States show that daily intake of 15-20 g olive oil reduced the risk of T2D by 13% [26]. In addition, olive oil intake attenuated hyperglycemia in patients with T2D [26]. Extra virgin olive oil (EVOO)-enriched meal significantly increased postprandial GLP-1 concentration in humans [27]. In addition, polyphenols extracted from olive leaf were found to improve glucose tolerance and insulin sensitivity in middle-aged overweight men [264], suggesting the phenolic compounds exert anti-diabetic effect. Oleuropein, ligstroside, oleuropein aglycone, hydroxytyrosol, tyrosol, and elenolic acid (EA) are the main phenolic components in olive, olive oil or olive leaf, of which has been reported to show anti-diabetic actions. Specifically, oral intake of oleuropein (20 mg) promoted the postprandial insulin and GLP-1 secretion in healthy individuals [33]. Oral administration of 10 mg/kg hydroxytyrosol reduced hyperglycemia in the diet-induced obese mice and db/db mice [265]. For my dissertation research, I found that elenolic acid (EA), a phenolic small molecule that is present in mature olive and EVOO, stimulates GLP-1 secretion in clonal L-cells and primary ileum crypts (Chapter 3). However, the *in vivo* effect of EA has not be identified. Given the important role for GLP-1 in blood glucoregulation, I investigated whether EA has anti-diabetic potential using diet induced obese (DIO) T2D mice.

## Materials and Methods

### Chemicals

Elenolic acid (EA) was generated from oleuropein and the quality of EA was determined by high resolution mass spectrometry as described in Chapter 3 of this dissertation; Diprotin A, TRI reagent, phenol red, methylcellulose, protease and phosphatase inhibitors, corn oil, triglyceride colorimetric kit were from Sigma (St. Louis, MO); high-capacity cDNA Reverse Transcription Kit and Power SYBR™ Green PCR Master Mix were from Applied Biosystems (Grand Island, NY); primers were synthesized by Eurofins Genomics (Louisville, KY); DC™ protein assay kit and TGX™ FastCast™ acrylamide kit were from Bio-Rad Laboratories (Hercules, CA); glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) antibodies were from Santa Cruz Biotechnology (Dallas, Texas); mouse monoclonal to β-actin antibody was from Abcam (Cambridge, MA); goat anti-rabbit antibody and SuperSignal™ West Femto substrate were from ThermoFisher Scientific (Waltham, MA); and goat anti-mouse antibody and Metabolism Multiplex Assay were from Millipore (Burlington, MA); alanine aminotransferase kit from Cayman (Ann Arbor, MI); free fatty acid assay was from Bioassay System (Hayward, CA); mouse insulin, leptin, adiponectin, GLP-1 ELISA kits were from Crystal Chem (Elk Grove Village, IL); glucose meter was from AgaMatrix (Salem, NH); Amplex™ Red Glucose kit was from Invitrogen (Waltham, MA).

### Animal studies

All procedures performed in animal studies were approved by animal care and use committee at Virginia Tech. C57/BL6J male mice were purchased from The Jackson Laboratory (Bar Harbor,

ME). All mice were kept under constant temperature (23ºC) and light cycle (12 h light/12 h dark) with *ad libitum* access to food and water.

**Experiment 1. Acute effect of EA on GLP-1 secretion**

For determining whether EA stimulates GLP-1 secretion *in vivo*, sixteen 10 weeks old male mice were randomly divided into two groups (n= 8/group). Mice were fasted 5 h and oral given vehicle or 50 mg/kg EA, and refed standard diet for 15 min. Blood samples were obtained after being fasted for 5 h in the pre-chilled tubes containing 50 μM diprotin A and 5 mM EDTA. After 15 min post-gavage, another batch of blood sample was drawn, and concentrations of total GLP-1 (tGLP-1) in plasma were measured following manufacturer' protocol (Crystal Chem).

**Experiment 2, Chronic effects of EA in middle-aged obese diabetic mice**

As T2D most often occurs in middle-aged adults, 10-mo-old obese mice were used to investigate the anti-diabetic effects of EA, as this age of the mouse is about equivalent to middle age in humans [266]. To this end, eighteen male mice (28-wk old) were fed a diet containing 58% kcal from fat (HFD, Research Diets, New Brunswick, NJ) for 6 mos to induce obesity and glucose intolerance. Afterwards, mice were divided into two groups with similar body weight and blood glucose each and then given either EA via oral gavage (50 mg/kg once daily) or vehicle for 7 wks. Ten age-matched mice fed standard chow diet (SD) were used as healthy control and were orally given vehicle for 7 wks. Body weight, food intake, non-fasting blood glucose, and fasting blood glucose were recorded weekly. Body composition of mice were evaluated by NMR Lean/Fat Analyzer for small animals (Bruker, Billerica, MA) at the beginning and end of the study.

**Glucose tolerance test (GTT), insulin tolerance test (ITT), and pyruvate tolerance test (PTT)**

For i.p.GTT, mice were fasted for 15 h and then given EA (50 mg/kg via gavage) or vehicle. After 30 min, mice were administered 1 g/kg glucose via i.p. injection, and blood glucose was measured at time -30, 0, 15, 30, 60, and 120 min before or after glucose injection using a glucose meter (AgaMatrix). For OGTT, mice were fasted for 15 h and then given a solution containing EA and 2 g/kg glucose or vehicle and 2 g/kg glucose via oral gavage for mice with a standard diet, but substitute to 1g/kg glucose for mice with a HFD diet. Blood glucose was measured at time 0, 15, 30, 60 and 120 min before or after oral gavage using a glucose meter (AgaMatrix). For PTT, mice were fasted for 15 h and then given 1 g/kg pyruvate via i.p injection. Blood glucose levels were measured at 0, 15, 30, 60, and 120 min post injection. For ITT, mice were fasted for 5 h and then injected (ip) 0.75 U/kg insulin. Blood glucose was measured at 0, 15, 30, 60, and 120 min post-injection of insulin.

**Fat tolerance test**

Mice were fasted overnight and then given 10 µl/g body weight of corn oil by oral gavage. Blood samples were obtained after 0, 1.5, 3, and 6 h after oral gavage. The plasma triglyceride concentrations were measured using the triglyceride colorimetric kit (Sigma).

**Plasma biochemical analyses**

Plasma alanine aminotransferase (ALT) was measured using a colorimetric kit (Cayman). Plasma insulin, leptin, and adiponectin were measured using mouse ELISA kits (Crystal Chem)). A homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as previous reported [267]. Plasma free fatty acid was measured using a free fatty acid assay (Bioassay System)

and plasma triglycerides were measured using a triglyceride colorimetric kit (Sigma). Liver triglyceride extraction was performed as previous reported [268]. The contents of hepatic triglycerides were measured using the same triglyceride colorimetric kit.

**Fecal lipid measurement**

Feces were collected for 3 consecutive days at 6th wk of the treatment. Feces were dried and weighed and extracted in chloroform-methanol (2:1) solution. After centrifugation, chloroform phase was collected for lipid measurement as previously described [269].

**Histological analysis**

Livers were collected, weighed, and fixed in 4% paraformaldehyde after euthanasia of the mice. Embedding and sectioning of liver samples were performed by AML Laboratories Inc. (Jacksonville, FL, USA). Tissue sections were de-paraffinized with xylene, rehydrated in graded ethanol solutions, and hematoxylin and eosin (H&E) staining was performed as previously described [270].

**Quantitative real-time PCR (qPCR) analysis**

Hypothalamus and livers were collected immediately after mice were euthanatized. Total RNA was isolated using TRI reagent. RNA (1 µg/sample) was reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit following the manufacturer's protocol. Quantitative PCR was preformed using 7900HT Fast Real-Time PCR System (Applied Biosystems) with Power SYBR™ Green PCR Master Mix with Gapdh as an internal control. A melting curve analysis was added to each sample to verify the non-specific amplification of the primers. The relative gene

expression levels were calculated by the $2^{-\Delta\Delta CT}$ method as described previously [231]. All samples were run in triplicates using the following protocol 5 min 96°C, 40× (10 s 95°C, 20 s 58°C, 20 s 72°C). The amplification efficiency of all primers in this study was between 90% and 110%. The sequences of the primers of each gene including the Gene ID from NCBI were listed in Table 1.

*Table 1. Primers for qPCR analysis*

| *Gene ID* | *Gene name* | | *Primer sequence (5'-3')* |
|---|---|---|---|
| *12896* | Carnitine palmitoyltransferase 2 (Cpt2) | Forward | CAACTCGTATACCCAAACCCAGTC |
| | | Reverse | GTTCCCATCTTGATCGAGGACATC |
| *12894* | Carnitine palmitoyltransferase 1a (Cpt1a) | Forward | CCATCCTGTCCTGACAAGGTTTAG |
| | | Reverse | CCTCACTTCTGTTACAGCTAGCAC |
| *26897* | Acyl-coenzyme A thioesterase 1 (Acot1) | Forward | GACAAGAAGAGCTTCATTCCCGTG |
| | | Reverse | CATCAGCATAGAACTCGCTCTTCC |
| *11430* | Peroxisomal acyl-coenzyme A oxidase 1 (Acox1) | Forward | ACGCCACTTCCTTGCTCTTC |
| | | Reverse | AGATTGGTAGAAATTGCTGCAAA |
| *56690* | Malonyl-CoA decarboxylase (Mlycd) | Forward | CTCGGGACCTTCCTCATAAAGAGA |
| | | Reverse | GAATAGTTCGTTCCTCCCATGCTC |
| *11370* | Long-chain specific acyl-CoA dehydrogenase (Lcad) | Forward | TTTCCTCGGAGCATGACATTTT |
| | | Reverse | GCCAGCTTTTTCCCAGACCT |
| *11364* | Medium-chain specific acyl-CoA dehydrogenase (Mcad) | Forward | AACACTTACTATGCCTCGATTGCA |
| | | Reverse | CCATAGCCTCCGAAAATCTGAA |
| *97212* | | Forward | TGCATTTGCCGCAGCTTTAC |

| | | | |
|---|---|---|---|
| | Trifunctional enzyme subunit alpha (Hadha) | Reverse | GTTGGCCCAGATTTCGTTCA |
| *14081* | Long-chain-fatty-acid--CoA ligase 1 (Acsl1) | Forward | ATCTGGTGGAACGAGGCAAG |
| | | Reverse | TCCTTTGGGGTTGCCTGTAG |
| *14433* | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Forward | TGTGTCCGTCGTGGATCTGA |
| | | Reverse | CCTGCTTCACCACCTTCTTGA |
| *104112* | ATP-citrate synthase (Acly) | Forward | AGGAAGTGCCACCTCCAACAGT |
| | | Reverse | CGCTCATCACAGATGCTGGTCA |
| *107476* | Acetyl-CoA carboxylase 1 (Acaca) | Forward | GGGCACAGACCGTGGTAGTT |
| | | Reverse | CAGGATCAGCTGGGATACTGAGT |
| *14104* | Fatty acid synthase (Fasn) | Forward | CCCTTGATGAAGAGGGATCA |
| | | Reverse | ACTCCACAGGTGGGAACAAG |
| *170439* | Elongation of very long chain fatty acids protein 6 (Elovl6) | Forward | CCCGAACTAGGTGACACGAT |
| | | Reverse | CCAGCGACCATGTCTTTGTA |
| *20249* | Acyl-CoA desaturase 1 (Scd1) | Forward | ACCTTCTTGCGATACACTCTGG |
| | | Reverse | GTAGTTGTGGAAGCCCTCGC |
| *12491* | Fatty acid translocase (Cd36) | Forward | GGCCAAGCTATTGCGACAT |
| | | Reverse | CAGATCCGAACACAGCGTAGA |
| *20787* | Sterol regulatory element binding transcription factor 1 (Srebf1) | Forward | GATCAAAGAGGAGCCAGTGC |
| | | Reverse | TAGATGGTGGCTGCTGAGTG |
| *19016* | Peroxisome proliferator-activated receptor gamma (Pparg) | Forward | ATTGAGTGCCGAGTCTGTGG |
| | | Reverse | GCAAGGCACTTCTGAAACCG |

| | | | |
|---|---|---|---|
| *58805* | Carbohydrate-responsive element-binding protein (Chrebp) | Forward | CGACACTCACCCACCTCTTC |
| | | Reverse | TTGTTCAGCCGGATCTTGTC |
| *18976* | Pro-opiomelanocortin (Pomc) | Forward | GACACGTGGAAGATGCCGAG |
| | | Reverse | CAGCGAGAGGTCGAGTTTGC |
| *11604* | Agouti-related protein (Agrp) | Forward | GTGTTCTGCTGTTGGCACTG |
| | | Reverse | ACTTCTTCTGCTCGGTCTGC |
| *109648* | Neuropeptide Y (Npy) | Forward | CACCAGACAGAGATATGGCAAGA |
| | | Reverse | TTTCATTTCCCATCACCACATG |

**Western blot analysis**

Liver samples were lysed in 20 mM Tris-HCl buffer (PH 7.4) containing 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, and 1% Triton supplemented with protease and phosphatase inhibitors and homogenized with bullet blender (Next Advance, Inc). Protein content in cell lysates was measured using a DC™ protein assay kit. Western blot gel (10%) was prepared according to the instruction of TGX™ FastCast™ acrylamide kit. Isolated proteins (20 µg/sample) were subjected to Western blot analyses by using standard procedures. Membranes were incubated with antibodies against glucose 6-phosphatase (G6Pase) (1:1000), phosphoenolpyruvate carboxykinase (PEPCK) (1:1000), and loading control GAPDH (1:5000) at 4ºC overnight. After wash, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:5,000) or anti-rabbit (1:125,000) antibodies for 1 h. The immunoreactive proteins were detected by using SuperSignal™ West Femto substrate. The optical density of protein band was quantitated with a software program (Image J, NIH) and normalized to that of the loading control.

**Experiment 3, Acute effects of EA on food intake, gastric emptying rate, and hormone secretion in mice**

Forty-eight 10-wk-old male mice were randomly divided into two groups. Group one was fed HFD (58% kcal from fat; Research Diets, New Brunswick, NJ) for 6 wks; group two was fed a standard chow-diet (SD). For food intake measurement, overnight-fasted mice were orally administered with vehicle or 50 mg/kg EA and then refed with SD or HFD. The food intake was measured at 0, 1, 3, 9, 21, 24 h after oral gavage of EA or vehicle. For measuring circulating GIP and PYY levels, overnight-fasted mice were given vehicle or 50 mg/kg EA, and then refed with SD or HFD for 1h. Blood was drawn from each mouse after overnight-fasting and 1 h of refeeding, and collected into 1.5 ml tubes coated with 5 mM EDTA and 10 μM diprotin A. Plasma was isolated after centrifugation at 3,000 rpm for 15 min at 4 ℃ and then stored at -80℃ for hormone measurements. For assessing gastric emptying rate, phenol red was administered, which is a widely used indicator for assessing stomach emptying rate [271]. To that end, mice were fasted for 5 h and then orally administered with vehicle or 50 mg/kg EA in a solution containing 2% methylcellulose and 5 mg/ml phenol red. Four mice from each group were euthanized at 0 and 20 min after oral gavage. The stomach was quickly removed, and its content was poured into 15 ml tube, and the stomach was washed thoroughly with 4 ml warm PBS. Afterwards, 2 mL 1M NaOH was added into each tube and measured the absorbance at 560 nm. The emptying rate of stomach was determined by measuring the absorbance at 560 nm of stomach content at 0 min and 20 min after oral gavage, and calculated using the equation:

(1- Abs (phenol red recovered after 20 min)/Abs (phenol red mixture)) %

**GIP and PYY assay**

For evaluating whether EA affects GIP and PYY release *in vivo*, SD and HFD mice were fasted for 15 h and then received 50 mg/kg EA or vehicle via oral route. Afterward, mice were refed for their respective diet for 1h. Blood samples drawn after fasting and refeeding were collected into 1.5ml tubes coated with 5 mM EDTA and 10 μM diprotin A and centrifuged at 3,000 rpm for 15 min at 4 ℃. Plasma was obtained and stored at -80 ℃ for measuring GIP and PYY concentrations according to the manufacturer's protocol (Millipore-Sigma).

**Gluconeogenesis assay**

HepG2 cells were seeded into 12-well plates with the concentration of $10^5$ cells/mL. Cells were cultured in serum-free DMEM supplemented with 5.5 mM glucose and 1% penicillin streptomycin for 5 h. Cells were washed with PBS and incubated in the gluconeogenesis medium (Phenol red-free DMEM containing 20 mM sodium lactate and 2 mM sodium pyruvate) with various concentrations of EA or forskolin (F, 10 μM) for 3 h. Glucose released into supernatants was measured using a glucose assay kit (Invitrogen) according to the manufacturer's protocol. Glucose level was normalized to the protein content in the sample.

**Statistical analysis**

Data were analyzed by unpaired student's t-test or one-way ANOVA where appropriate, followed by Duncan's multiple range test using JMP 13 (SAS Institute Inc, Cary, NC). The $p < 0.05$ was considered significant difference. Values are presented as mean ± standard error of mean (SEM).

## Results

### Acute administration of EA augmented plasma GLP-1 concentrations in mice

As previous study (Chapter 3) showed that EA stimulates GLP-1 secretion from cultured GLP-1 secreting cells and ileum crypts, I further investigated whether acute oral EA administration also increases GLP-1 secretion *in vivo.* To that end, mice were fasted for 5 h and then given EA (50 mg/kg, oral gavage) or vehicle. Blood were sampled at 0 min and then 15 min after EA administration. As expected, basal blood GLP-1 concentrations were similar between control and EA group. However, mice with EA administration had significant higher postprandial GLP-1 concentrations (from 9.2 ± 2.0 pM to 13.6 ± 2.2 pM) as compared to the control mice (from 8.7 ± 2.5 pM to 7.7 ± 2.0 pM) (Fig. 1A), suggesting that EA stimulates GLP-1 secretion.

As the next aim was to investigate whether a single dose of EA administration improves oral glucose tolerance, mice were fasted overnight and orally given a mixture of EA (50 mg/kg) and glucose (2 g/kg) or vehicle and glucose (2 g/kg) for SD mice, but orally given a mixture of EA (50 mg/kg) and glucose (1 g/kg) or vehicle and glucose (1 g/kg) for HFD mice. Blood glucose was measured at 0, 15, 30, 60, and 120 min after oral gavage. There was no significant difference of glucose tolerance between vehicle and EA in SD-fed mice (Fig. 1B). However, EA mice fed HTD exhibited an improved glucose tolerance as compared with the control mice ($P < 0.05$) (Fig. 1C).

### EA was a potent anti-diabetic agent in DIO mice

As mouse at 10-14 months old is roughly equivalent to 38-47 old humans [266], and the incidence of T2D increases in middle-aged adults, older adult (28 wks) mice were fed a HFD for 24 wks when mice became obese with insulin resistance and glucose intolerance as confirmed by

performing ITT and GTT (data not shown). The mice were then treated with vehicle or 50 mg/kg for 7 wks. As shown in Fig. 2, the fasting blood glucose levels of EA group exhibited a significant decrease compared to that of HFD group as early as 3-wk after treatment (Fig. 2A). Mice with EA administration had a significantly lower non-fasting blood glucose at the end of 2-wk treatment as compared to HFD, but this difference disappeared at the end of 4-wk treatment (Fig.2B). In addition, EA-treated mice showed normalized glucose tolerance to the comparable levels in SD mice (Fig. 2C-E). Mice treated with EA showed an increased insulin tolerance, as compared to the HFD mice (Fig. 2F and G), suggesting that EA improved insulin sensitivity. Consistently, mice treated with EA had significantly lower HOMA-IR than that of obese control mice (Fig. 2H). The GSIS was performed for assessing the insulin secretion in response to glucose stimuli. The blood samples were obtained at 0 and 30 min after glucose injection. As expected, HFD mice had significantly higher fasting insulin concentrations in the circulation as compared with those in SD mice, but EA greatly mitigated hyperinsulinemia in obese mice (Fig. 2I). There was no significant difference of insulin concentration between HFD and EA group at 30 min after glucose injection. However, insulin concentration at 30 min was higher than that at 0 min in HFD ($P < 0.05$) and EA ($P = 0.07$) group, suggesting that pancreatic β-cells were sensitive to glucose stimuli.

**EA ameliorates obesity in DIO mice**

Oral administration of EA significantly reduced the body weight of DIO mice through the time course of the treatment (Fig. 2J). As aforementioned in the methods part, mice were fed HFD for 6-mo before treatment, and body weight of mice within the timeframe of HFD feeding was robustly increased as compared to that of SD mice. The beginning body weight of SD, HFD, EA-treated mice before treatment was 38.56 ± 1.83, 53.56 ± 0.91, and 53.02 ± 1.00 g, respectively.

EA administration for 1 wk already reduced body weight by 5.4 ± 1.4% compared to the beginning body weight (P< 0.001), which was steadily decreased over the course of the 5-week of treatment period. At the end of 5-wk of treatment, DIO mice given EA weighed 47.3 ± 1.01 g, equivalent to 10.7± 1.6% weight loss as compared with their initial body weights (P< 0.001), whereas the body weight of vehicle-treated control mice was continuously increased by 9.2 ± 2.2% (P<0.001). Consistently, the result of body composition analysis showed that EA significantly reduced fat mass as well as the percentage of the fat mass (3.9 %) while marginally increased lean mass in obese mice relative to the HFD mice (Fig. 2K). Cumulative food intake of EA-treated mice at the first week of treatment was 11.9 ± 0.6 g/mouse, while the vehicle-treated control obese mice consumed 17.7 ± 0.7 g/mouse during the same period (p<0.001), a reduction of 32.8 % in food intake that was roughly steady throughout the feeding experiment. At the end of 5-wk treatment, cumulative food intake for the control group was 94.1 ± 1.4 g/mouse, which was reduced to 73.5 ± 3.6 g/mouse in EA-treated mice, corresponding to a 21.9 % reduction relative to the control mice, suggesting the body weight loss was predominantly due to the reduced food intake in EA-treated group (Fig. 2L).

To investigate whether EA reduces food intake in an acute or accumulative manner, I measured the food intake at 1, 3, 6, 9, 21 and 24 h after administration of vehicle or EA via gavage (Fig. 2M). The reduction in food intake was the most significant during the first hour following EA administration. During this time, vehicle-treated mice fed with HFD consumed 1.1 ± 0.2 g per cage while EA-treated mice only ate 0.5 ± 0.1 g per cage (p<0.05). However, the significant hypophagic effect of EA only persisted for 3 h following EA treatment and no significant differences in food intake were observed thereafter. Consistently, EA also suppressed food intake in SD-fed mice with a similar inhibitory pattern as detected in HFD-fed mice.

To determine whether EA-reduced food intake is associated with gastrointestinal transit rate, I assessed and compared gastric emptying rate in mice given vehicle or EA. Mice with EA administration reduced gastric emptying rate by about 50% after 20 min of oral administration (Fig. 2N), suggesting that EA delays the stomach movement, which might lead to the increased satiety and reduced the food intake.

**EA increased blood PYY and GIP levels**

In addition to GLP-1, several other gut hormones released in response to nutrients and energy intake, including peptide tyrosine tyrosine (PYY) and glucose-dependent insulinotropic polypeptide (GIP), also play a role in regulating gut mobility, satiety, and food intake in both humans and rodents [272, 273]. As EA administration led to a reduction in food intake, next aim was to investigate whether EA regulates the secretion of these anorexigenic hormones. In that regard, overnight-fasted SD and HFD mice were orally given vehicle or 50 mg/kg EA and refed respective diet for 1 h. Blood samples were obtained after fasting and refeeding and measured the GIP and PYY concentration. As expected, refeeding GIP was higher than fasting GIP both in SD and HFD mice (Table 2). Oral EA administration significantly potentiated refeeding GIP concentration both in SD (from 25.8 ± 9.7 pg/ml to 437.9 ± 80.3 pg/ml, $P < 0.05$) and HFD (from 48.6± 8.5 pg/ml to 505.8± 131.1 pg/ml, $P< 0.05$) mice. However, neither vehicle-treated SD nor HFD mice exhibited significantly increased refeeding GIP concentration. In addition, the fasting GIP levels was higher in HFD mice as compared to those in SD mice, but the difference is not statistically significant. The postprandial GIP was increased in SD and HFD mice with EA administration, but the difference didn't reach statistical significance (due to the variation). In addition, oral EA administration increased the postprandial PYY secretion in HFD mice, from 13.2

± 4.0 pg/ml to 62.5 ± 18.4 pg/ml (P< 0.05). However, postprandial PYY concentrations in circulation were not significantly increased in neither vehicle-treated SD nor HFD mice as compared with their fasting levels. Similar to GIP, the fasting PYY was higher in SD mice than that in HFD mice, but the difference was not statistically different. Furthermore, the refeeding PYY was increased in SD and HFD mice after EA administration, but the difference was not statistically significant (Table 2).

*Table 2. Plasma GIP and PYY concentration in SD and HFD mice.*

| | | *GIP (pg/ml)* | | *PYY (pg/ml)* | |
|---|---|---|---|---|---|
| | | Fast | Refeed | Fast | Refeed |
| *SD* | C | 26.6±7.0 | 117.2± 43.7 | 31.0±18.9 | 6.9# |
| | EA | 25.8±9.7 | 437.9±80.3* | 29.1±13.1 | 137.6±61.0 |
| *HFD* | C | 55.3±18.0 | 237.2±73.7 | 13.2±6.4 | 12.5±5.7 |
| | EA | 48.6±8.5 | 505.8±131.1* | 13.2±4.0 | 62.5±18.4 * |

Data were shown as means ± SEM. n= 5 for SD group and 6 for HFD group. *, P< 0.05 vs. individual fast status. #, the lowest detected range of the kit.

Hypothalamus plays a crucial role in controlling food intake by producing the anorexigenic neuropeptides, proopiomelanocortin (Pomc)/cocaine and amphetamine-regulated transcript (Cart), and the orexigenic peptides, neuropeptide Y (Npy)/agouti-related peptide (Agrp) [194]. PYY and GLP-1 regulated appetite via activating the receptors in the hypothalamic arcuate nucleus (ARC) [274]. Given that EA induced GLP-1 and PYY secretion *in vivo*, EA may regulate the expression of appetite-associated genes in the hypothalamus. To this end, mouse hypothalamus was harvested in fasting and refeeding conditions from SD, HFD, and EA groups and Agrp, Npy, and Pomc expression levels quantified. As Agrp and Npy are orexigenic peptides, the relative expressions of

Agrp and Npy were higher in fasted mice relative to those in fed mice. On the contrary, Pomc mRNA abundance was higher in fed mice than that in fasted mice. As expected, EA-treated mice had a lower expression of Agrp in the refeeding condition than that of fasting condition, whereas the difference was not statistically significant (P= 0.06 Agrp in EA) (Fig. 2O and P). However, HFD mice did not have a lower expression of Agrp after refeeding. In addition, obese mice given EA has lower Agrp levels as compared to those of HFD following refeeding (P =0.05). However, EA did not alter hypothalamic Npy expression in mice during the refeeding period. Similarly, the expression of Pomc did not differ between EA treatment and the control as well as between fasted and refed mice (Fig. 2Q).

**EA reversed obese-associated alterations in plasma biochemical variables**

As EA induced weight loss and ameliorates obesity in DIO mice, I investigated whether EA administration reverses the HFD-mediated plasma biochemical alteration. As expected, the HFD mice had higher plasma free fatty acid (FFA) concentrations relative to those in SD mice (P< 0.01). However, mice treated with EA had plasma FFA levels comparable with those in SD mice, corresponding to a 41% reduction from those of HFD mice (P< 0.01) (Fig. 3A). Similarly, EA treatment reduced plasma triglyceride concentrations in obese mice (P< 0.05) (Fig. 3B). Leptin and adiponectin are two important hormones that are secreted from adipose tissues, and play a role in regulating metabolic homoeostasis [275]. As expected, DIO mice were had more plasma leptin as compared with SD mice (P< 0.01). However, treatment with EA significantly reduced the elevated plasma leptin levels in DIO mice (Fig. 3C). However, neither HFD mice nor EA mice had a significant difference in plasma adiponectin as compared to SD mice (Fig. 3D).

**EA restored the impaired lipid metabolism in DIO mice**

As EA-treated mice displayed significantly lower plasma FFA and triglyceride levels in the circulation of DIO mice, I examined whether EA reduces intestinal lipid absorption, thereby contributing to the effects EA on plasma lipid profile in DIO mice. To this end, I first measured the fecal lipid contents and found that EA administration significantly increased fecal triglyceride contents as compared with the control mice (Fig. 4A). Next, I performed the fat tolerance test by giving a bolus of corn oil via oral gavage and then measured plasma triglyceride concentrations at 0, 1.5, 3, and 6 h after oil administration. As expected, the plasma triglyceride levels were barely changed after oil challenge in EA-treated mice (Fig. 4B and C), whereas plasma triglyceride concentrations were increased by 2-fold at 1.5 h and 3 h in response to oil challenge. Combined, these data suggest that EA might improve lipid profile in circulation partially via reducing fat absorption.

Liver is an important organ for lipid metabolism [276]. The livers of obese mice were a pale color and oversized as compared with that of SD and EA-treated mice (Fig.4D). In addition, the fresh liver weight of HFD mice was much heavier (2-fold) than that of SD and EA-treated mice (Fig. 4D), whereas the liver weight of EA-treated mice was comparable to that of SD mice. The increased liver weight in the HFD mice might result from the increased liver lipid contents. Consistently, histological staining (H&E) showed that the liver from HFD had more intracellular lipid droplets than that of EA or SD group (Fig. 4E), suggesting that EA largely reversed lipid accumulation caused by HFD. Triglyceride contents in the frozen liver samples were measured. The result showed that the HFD mice accumulated more hepatic triglycerides, but EA-treated mice had much lower hepatic triglyceride contents, which were comparable as those in SD mice (Fig. 4F), suggesting that EA reversed the fatty liver induced by constant HFD feeding. Next, ALT

activity was measured for evaluating the function of liver (Fig. 4G). As expected, HFD mice had an extremely high plasma ALT activity, 50.6 ± 7.7 U/L, whereas EA administration reduced plasma ALT activity to 20.7 ± 2.6 U/L, corresponding to a 60% reduction as compared to that of HFD ($P < 0.001$).

To determine whether EA treatment directly restores the impaired hepatic lipid metabolism caused by chronic HFD feeding, I performed qPCR to assess the relative expression levels of genes that are involved in fatty acid oxidation and lipogenesis. The hepatic expression levels of Cpt1, Cpt2, Acot1, Acox1, Lcad, Mcad, and Hadha were significantly lower in HFD mice relative to those in SD mice (Fig. 4H), which were reversed by treatment with EA (Fig. 4H), suggesting that EA administration reversed the reduced fatty acid oxidation that was caused by a long-term HFD feeding in DIO mice. However, EA did not significantly alter the expression of the genes responsible for lipogenesis in obese mice (Fig. 4I), suggesting that EA might not modulate the lipogenic pathway in the liver of the DIO mice.

**EA suppressed gluconeogenesis in DIO mice**

During long term fasting, liver is the main organ for glucose production via gluconeogenic pathway. As the fasting blood glucose levels were significantly lower in EA-treated mice that those in HFD alone-fed mice, PTT assay was performed and showed that obese mice displayed higher plasma glucose levels during PTT relative to SD mice (Fig. 5A and B), suggesting that gluconeogenesis was abnormally increased in obese mice. However, EA treatment for 5 wks completely normalized gluconeogenesis. Consistently, the expression levels of the key enzymes (PEPCK and G6Pase) in the livers for gluconeogenesis were elevated in obese mice but was lower after EA treatment, with a reduction of 20% and 30%, respectively (Fig. 5C and D). To determine

whether EA directly suppresses glucose production, I cultured HepG2 cells in gluconeogenesis medium in the presence or absence of EA and then measured glucose production. The result showed that EA dose-dependently reduced glucose production (Fig. 5E), confirming that EA directly suppresses hepatic gluconeogenesis.

## Discussion

While previous studies have extensively explored naturally occurring polyphenols that have anti-obesity and anti-diabetic properties, limited polyphenols possess the dual actions. Indeed, to the best of our knowledge, only celastrol, that isolated from *Tripterygium wilfordii*, exerts its anti-diabetic and anti-obesity action via leptin receptor signaling [198]. In the present study, I found that a small molecule EA, which is generated by enzymatic hydrolysis of oleuropein, is a novel GLP-1 agonist and improves glycemic control in middle-aged DIO mice. In addition, EA reduced body weight via effectively decreasing food intake, as a result of the delayed gastric emptying rate. Given GLP-1 reduces food intake via delaying gastric emptying rate, the weight control effect of EA might result from the activation of GLP-1. It is known that middle-aged and older adults have the highest risk for developing T2D [1]. Therefore, the results from the current study using middle-aged rodent models might be more relevant to humans.

Acute administration of EA elevated plasma GLP-1 concentrations in mice, demonstrating that EA-evoked incretin secretion from L-cells *in vitro* is recapitulated *in vivo*. Consistent with GLP-1-mediated action, acute oral administration of EA significantly improved glucose tolerance in mice. These results strongly suggest a metabolically beneficial effect of EA. In addition to its important role in maintaining glycemic homeostasis through promoting β-cell function [7-9], GLP-1 also was found to delay stomach emptying and induce satiety in animal models of obesity

[73] and humans [277]. Therefore, the result that acute EA administration reduced stomach emptying rate in obese mice is at least partially ascribed to its effect on GLP-1 secretion. In addition to GLP-1, several other gut hormones that are released in response to nutrient and energy intake, including PYY, GIP, and oxyntomodulin (OXM), also play a role in regulating gut mobility, satiety, and food intake in both humans and rodents [278]. Specifically, the gut hormone PYY, which is secreted from L-cells [279], is an anorectic hormone that regulates gut mobility and directly suppresses appetite in the brain via a Y2 receptor-mediated pathway [280]. It was found that mice deficient in PYY are hyperphagic and obese while PYY replacement restored their lean phenotype [279]. Therefore, PPY is an important gut hormone with anti-obesity and anti-diabetic effects. In the present study, oral gavage of EA to fasted mice followed by refeeding significantly elevated circulating levels of PYY and GIP. GIP is secreted from intestinal K-cell, and potentiated GLP-1 secretion in healthy subjects [281]. In addition, the GIP/GLP-1 dual agonist improved glycemic control and reduced body weight in the patients with T2D [282]. Thus, it is possible that these hormones also contribute to the overall hypophagic and anti-obesity actions of EA.

Given GLP-1 elevated glucose tolerance via enhancing the glucose disposal [283], the acute effect of EA on glucose tolerance is owing to the EA-induced GLP-1 secretion. Obese mice with EA administration exhibited improved insulin sensitivity and glycemic control, which might result from the reduced body weight and fat mass. EA-treated mice showed a 4% decrease in fat mass while the lean mass was increased by 4% in comparison with the obese control, which may contribute to the improved insulin sensitivity of EA-treated mice, as muscle content in the body is reversely associated with insulin resistance [284]. In addition, the chronic effect of EA on glucose tolerance in obese mice might be owing to the improve insulin sensitivity, as the improved insulin sensitivity leads to the enhancement of insulin action with improving insulin-dependent glucose

disposal [285]. In this regard, EA may be an effective oral GLP-1 agonist to improve insulin sensitivity and glucose tolerance in obese mice.

While liver is one of the main organs for triglyceride synthesis, a relatively small quantity of triglyceride is stored within the liver in the normal condition [286]. Triglyceride is a neutral storage form of fatty acids (FAs). The balance of hepatic triglyceride metabolism is primarily maintained by FAs acquisition via uptake from blood or *de novo* lipogenesis and triglyceride disposal via secretion into circulation as triglyceride-enriched very low-density lipoprotein or FA oxidation. However, hepatic triglyceride accumulation is increased in several diseases, like non-alcoholic fatty liver disease, as a result of the imbalance of triglyceride metabolism [287]. EA significantly reduced hepatic triglyceride accumulation and plasma triglyceride levels in DIO mice, indicating that EA sustains the balance of triglyceride metabolism. In addition, fecal lipid contents were elevated in EA-treated mice, suggesting that the beneficial effect of EA on liver and circulating lipid profile may partially due to the disruption of fat digestion and absorption, which was further confirmed by fat tolerance test, showing that EA indeed reduced triglyceride trafficking to the circulation. The *de novo* lipogenesis primarily occurs after a high carbohydrate-diet intake and utilizes substrate, like acetyl-CoA from Krebs Cycle, to generate fatty acids (FAs) [288]. The increased *de novo* lipogenesis is associated with the activation of the transcription factors, like sterol response element binding protein 1c (Srebp-1c) and carbohydrate response element binding protein (Chrebp) or their dependent lipogenic pathway [289]. Lipolysis occurs during fasting and involves the hydrolysis of triglycerides into glycerol and FAs. Most FAs are further catalyzed via mitochondrial β-oxidation route to generate fuel for cell utilization or ketone body. Furthermore, it is possible that EA directly promotes FFA oxidation in the liver, as it upregulated the critical genes responsible for fatty acid oxidation, thereby contributing to the reduced fat accumulation in

the liver. These results strongly demonstrate that the reduced fat accumulation in the liver and fat tissue in EA-treated mice is due to altered fat absorption and hepatic lipid metabolism. However, it is still unclear how EA directly exerts these effects or its effect on fat metabolism is simply due to the secondary action whereby it suppresses food intake.

Liver plays a vital role in glucose homeostasis via promoting glucose production in the fasting state and glucose store in the feeding state. The increased hepatic gluconeogenesis might attribute to hyperglycemia in T2D [290]. EA-treated mice had a better pyruvate tolerance and lower fasting blood glucose than HFD mice, which might result from the reduced expression level of two rate-limiting enzymes, PEPCK and G6Pase, in the gluconeogenic pathway. Administration of GLP-1 modulated hepatic lipid and glucose metabolism [291, 292], suggesting GLP-1 plays a role in reversing the aberrant glucose metabolism. Indeed, hepatic gluconeogenesis is precisely controlled by insulin and glucagon [293, 294]. Given GLP-1 stimulated insulin secretion and suppressed glucagon secretion [295, 296], thus, the inhibitory action on hepatic gluconeogenesis might be a result of the GLP-1 action. However, EA suppressed glucose production in the HepG2, suggesting EA might directly regulate hepatic glucose metabolism. As aforementioned, EA, as most dietary polyphenols, might first be metabolized in the intestine and further metabolized in the liver, but the amount of EA reaching in the liver is still unclear. In this context, the underlying mechanism of hepatic glucoregulatory and lipid regulatory effect of EA is needed further study.

While the safety of EA is largely unclear, EA is isolated from EVOO and mature olive, which were widely consumed by humans, suggesting that EA is conceivably safe. In this study, EA was given via oral gavage and effectively reduced food intake. It is worth noting that the altered food intake is not a consequence of the food palatability, given that EA is administered into the stomach directly, and thereby no palatability changes would be detected. In addition, EA-evoked

weight loss, accompanied with an obviously decreased percentage of fat mass, but the increased percentage of lean mass in DIO mice, suggesting that EA modulates body composition viaits anti-obesity role. Notably, the plasma ALT activity has been used as an indicator of liver function [297], which was improved in EA-treated mice. All these results illustrate that EA may be a safe agent for oral administration.

While I didn't perform a dose-dependent study due to the limited quantity of available EA for this study, the dose of EA in this study (50 mg/kg of body weight), which is equivalent to 284 mg/day for a 70 kg person based on a widely used conversion methods from animals to humans [201], could be realistically consumed by humans. However, EA dose-dependently induced GLP-1 clonal L-cells and primary ileum crypts (Chapter 3), suggesting the possibility of the dose-dependent effects of EA *in vivo*.

In summary, I found that an olive-derived molecule, EA, ameliorated the impaired glucose and lipid homeostasis in DIO mice, which may be due to promoting GLP-1 secretion, leading to the reduced food intake and weight loss, given that GLP-1 plays a central role in regulating food intake and delaying gastric emptying. In addition, EA might rectify the altered hepatic glucose and lipid metabolism in DIO mice. Therefore, the current results suggest that for the first time, as to the best of knowledge, EA is a naturally occurring, potent anti-obesity and anti-diabetic agent at least partially via activation of the GLP-1 signaling pathway.

## Figures

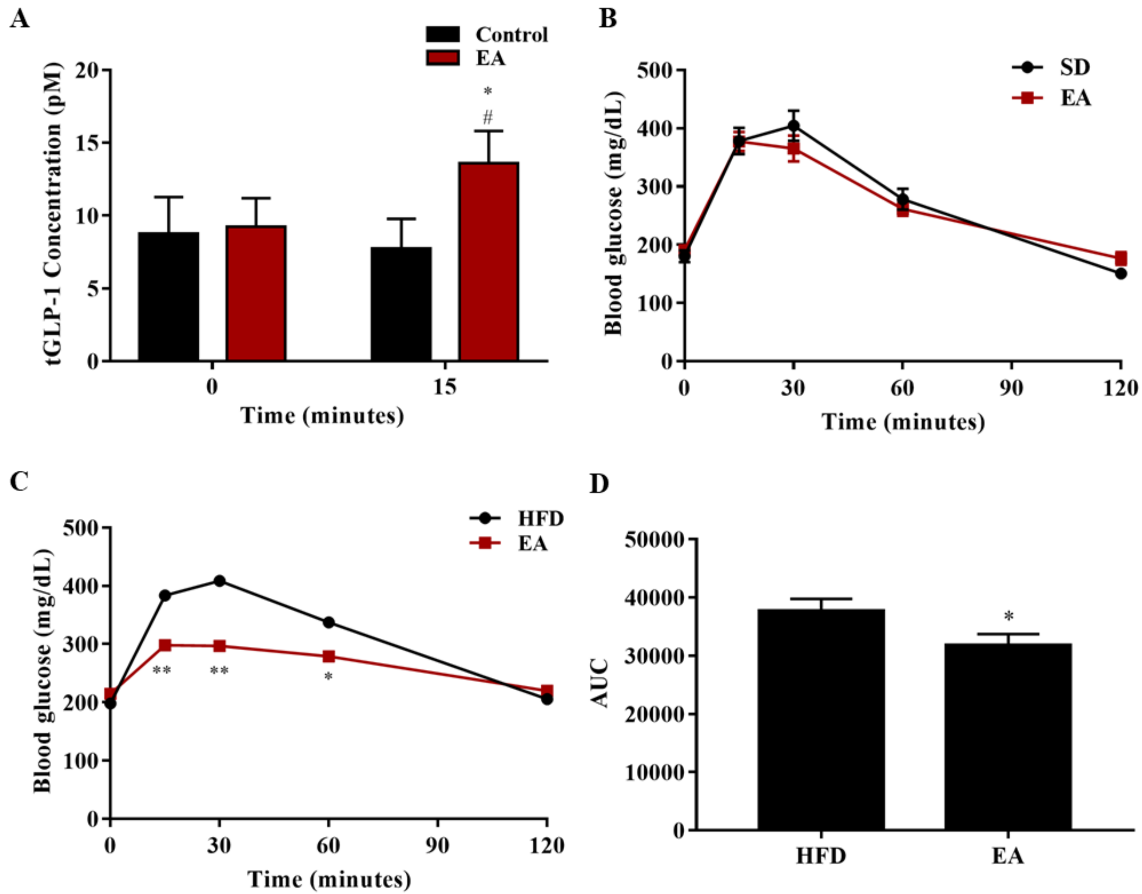


**Figure 1. Acute effect of EA *in vivo*.** (A) Vehicle or 50 mg/kg EA were orally given to the 5h-fasted mice. Blood samples were collected before oral gavage (Time 0) and after 15 min of oral gavage (Time 15) for measuring total GLP-1 (tGLP-1) concentration. Data were shown as means ± SEM; N =8 for each group, * $p < 0.05$ vs. time 0; # $p < 0.05$ vs. control. 28-wk-old SD mice (B) and 10-mo-old HFD mice (C) were fasted overnight and orally given mixture of vehicle or 50 mg/kg EA in 2g/kg glucose for SD mice and 1g/kg glucose for HFD mice. Blood glucose was measured after 0, 15, 30, 60, and 120 min after gavage. The Area under the curve of HFD mice was calculated and shown in D. Data were shown as means ± SEM; N = 9 for each group, * $p < 0.05$ vs control.

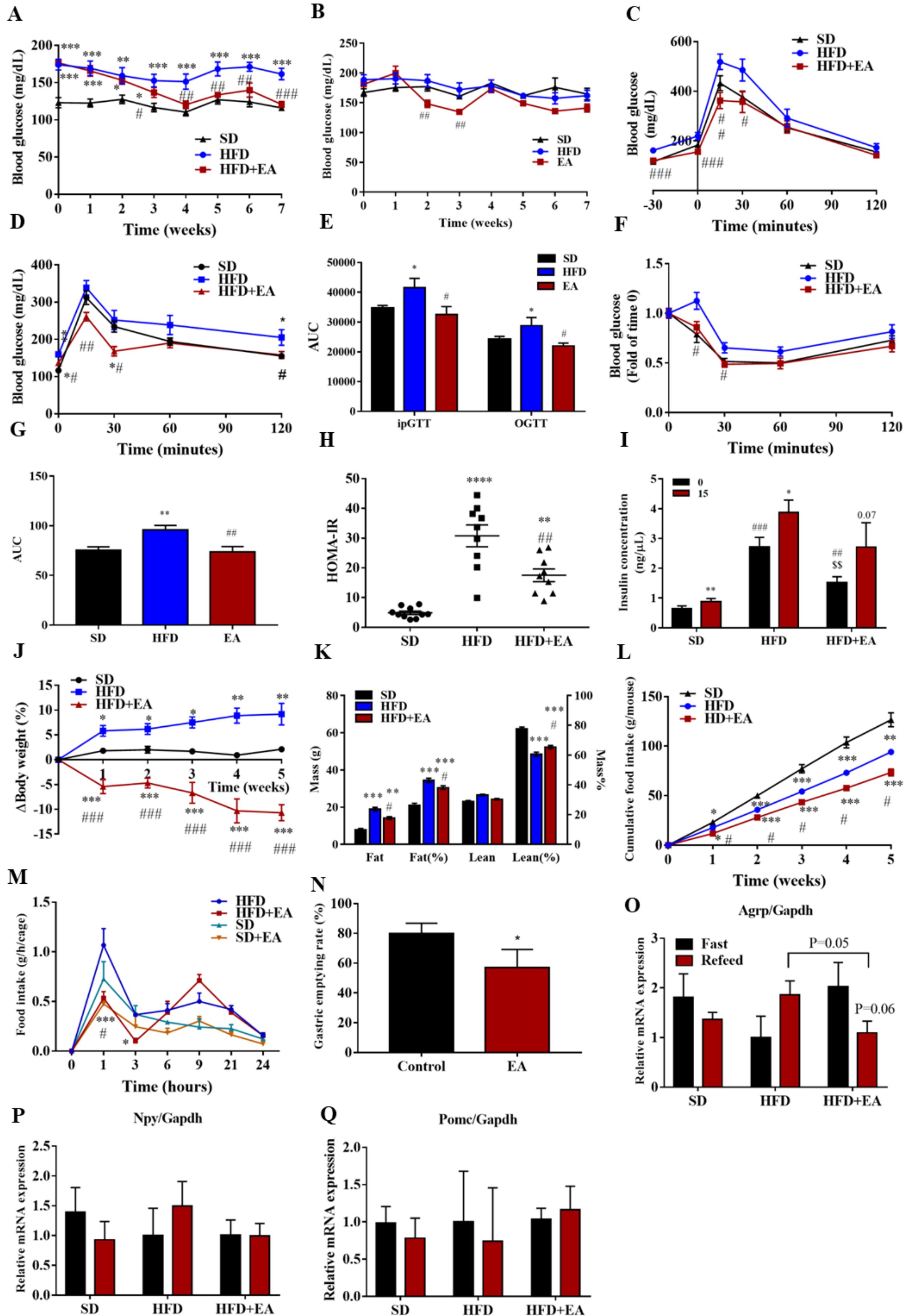

**Figure 2. The anti-diabetic and anti-obesity effect of EA in DIO mice.** SD and HFD mice were orally given 50 mg/kg EA or vehicle for 7 wks. Fasting blood glucose (A) and non-fasting blood glucose (B) were measured weekly during the treatment. i.pGTT(C) and OGTT (D) were performed after 5 wks and 6 wks of the treatment, respectively. The corresponding area under the curve of each test was calculated in E. ITT (F) was performed after 6-wk of the treatment, and the blood glucose was normalized to the beginning value of each group. Area under the curve of ITT was presented in G. HOMA-IR was calculated based on fasting glucose and fasting insulin concentration and shown in H. GSIS was performed with overnight-fast and given 1 g/kg glucose via i.p injection (I). Body weight changes were recorded weekly (J). Body composition (K) was measured at the end of the experiment. (L) Food intake of each group was recorded every week. SD and HFD mice were fasted and orally given vehicle or 50mg/kg EA. The food intake within 24 h was measured (M). Gastric emptying rate with EA administration was performed in SD mice (N). (O-Q) Hypothalamus was harvested and mRNA was isolated. Appetite-related neuropeptide mRNA expression was analyzed using qPCR. Data were presented as mean ± SEM; n= 10 mice of SD, 9 mice of HFD, and 9 mice of EA in the figure A-M; n= 12 (3 cages) for each treatment in figure N; n= 4 in figure O; n= 5 for SD, and 4 for HFD and EA in each condition in figure P-R. *, $P < 0.05$ vs. SD; #, $P < 0.05$ vs. HFD.

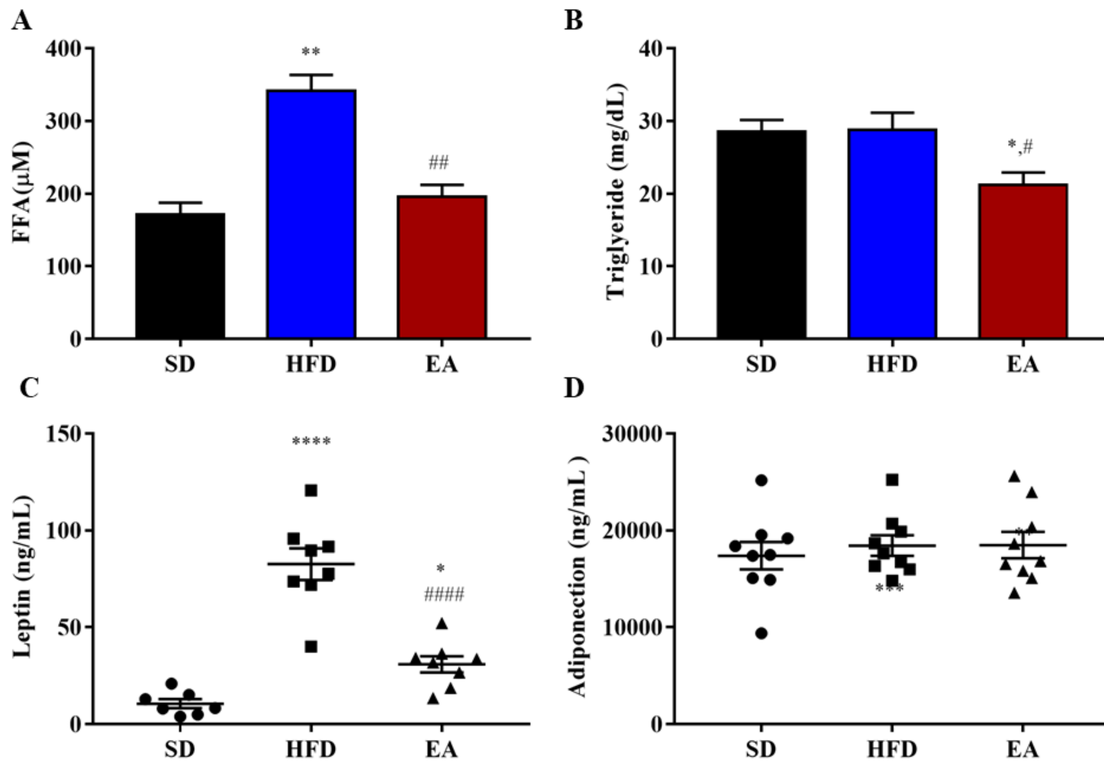


**Figure 3. EA reversed HFD-mediated plasma biochemical alteration.** Blood samples were obtained in the overnight-fast mice and stored at -80 ºC for free fatty acid (FFA, A), triglyceride measurement (B), leptin (C), and adiponectin (D). Data were presented as mean± SEM, * $P < 0.05$ vs. SD; # $P < 0.05$ vs. HFD.

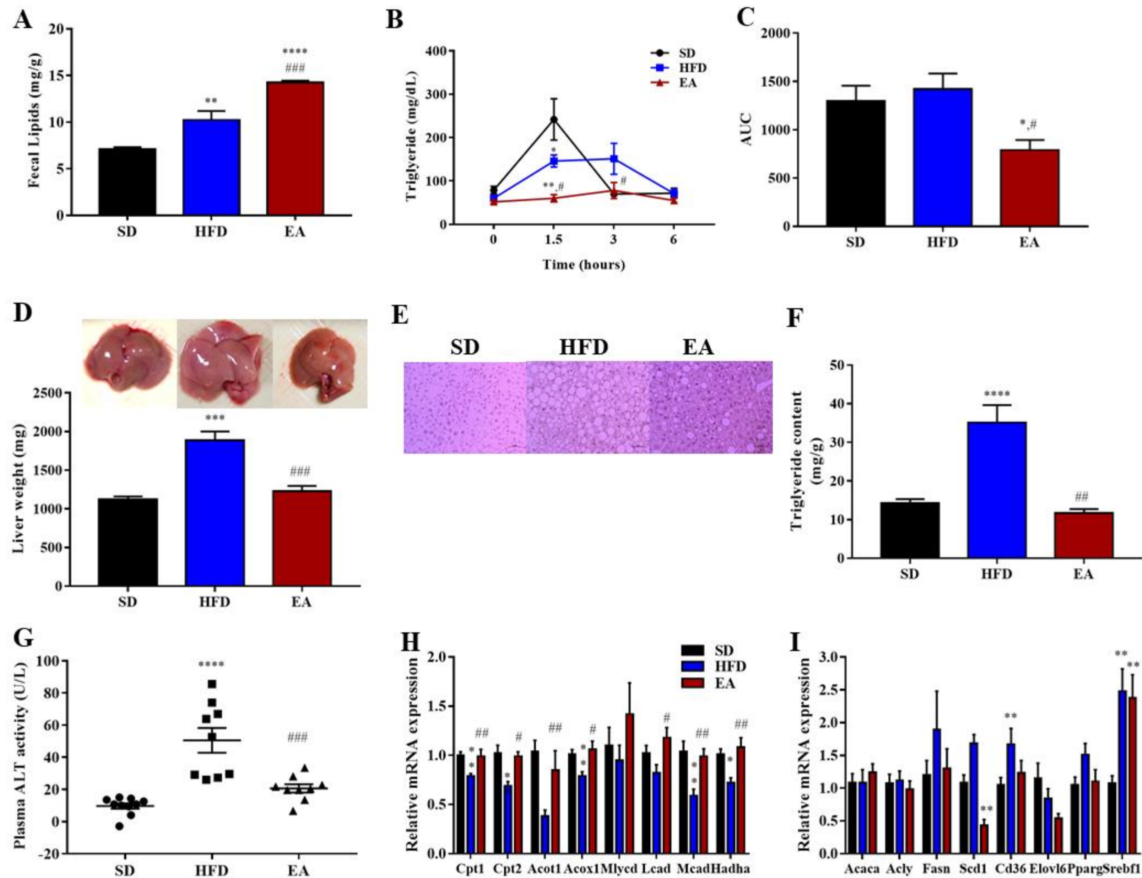


**Figure 4. EA restored lipid metabolism.** (A) Feces were collected and used for lipid content analysis. (B) Fat tolerance was performed using 8h-fast mice with corn oil gavage. Blood samples from 0, 1.5, 3, 6 h after oral gavage were obtained for measuring plasma triglyceride concentration. Area under the curve was calculated and shown in C. Morphology of liver and the weight of fresh liver (E) were assessed. (F) The triglyceride was extracted from frozen liver and measured with commercial kit. (G) Liver sections were stained with HE to examine the hepatocyte morphology. (I) Plasma ALT was assessed to analyze liver damage. qPCR was used to determine the expression of fatty acid β-oxidation (E) and lipogenic (F) genes in liver. All data were presented as mean ± SEM. *, $p < 0.05$ vs. SD; #, $p < 0.05$ vs. HFD.

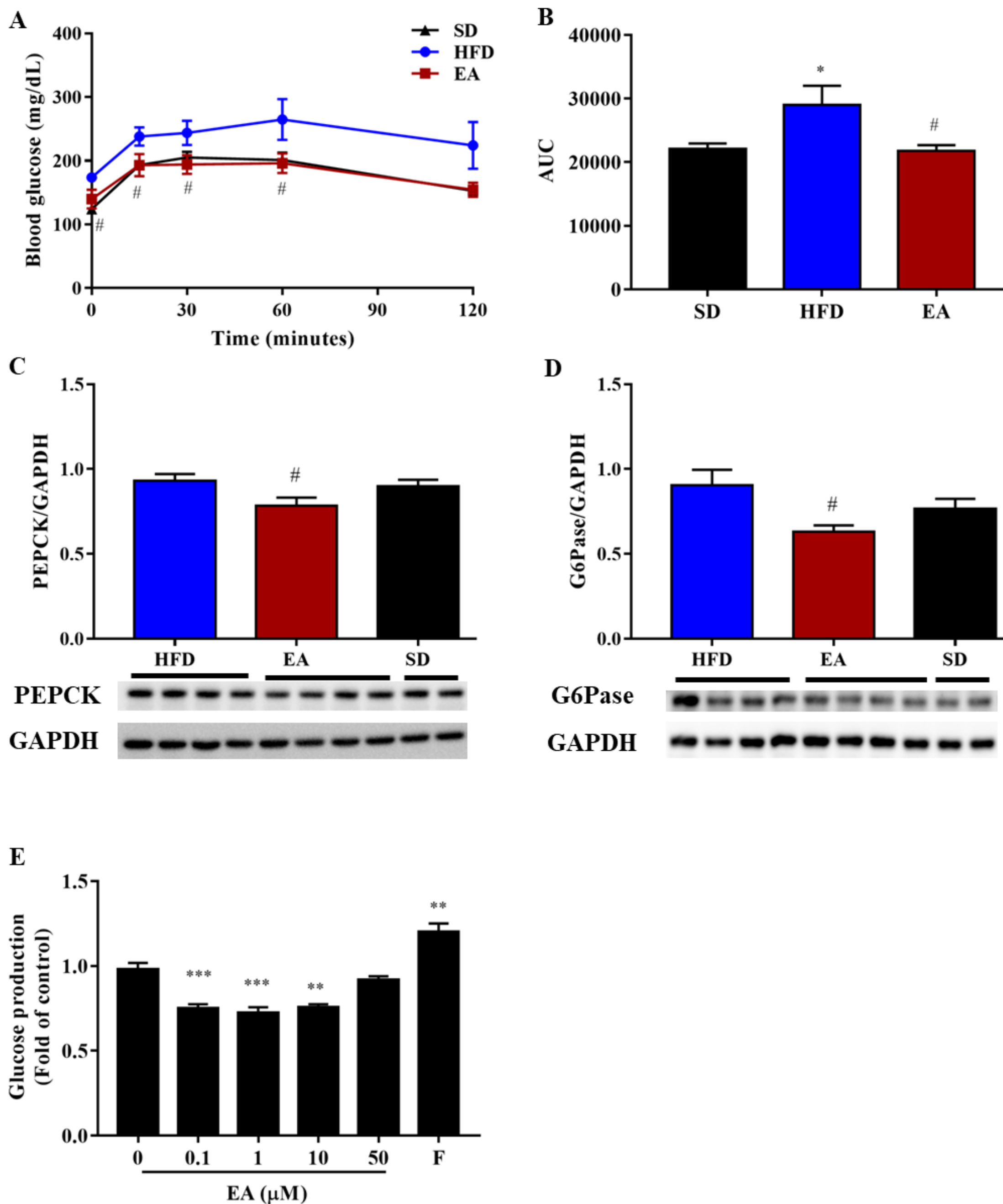


**Figure 5. EA suppressed hepatic gluconeogenesis.** (A) Pyruvate tolerance test (PTT) was performed after 6 wks of treatment. Mice were fasted overnight and given 1 g/kg pyruvate via i.p injection, and blood glucose was measured after 0, 15, 30, 60, and 120 min after injection. The corresponding area under the curve was calculated (B). Western blot analysis was performed to evaluate the protein expression level of gluconeogenic enzyme PEPCK (C) and G6Pase (D) in the liver lysates. All data were presented as mean ± SEM, #, $p < 0.05$ vs. HFD. (E) Glucose production was measured in HepG2 cell with 0, 0.1, 1, 10, and 50 µM EA or forskolin (F, 25 µM). All data were presented as mean ± SEM, #, $p < 0.05$ vs. control, n = 5.

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# CHAPTER FIVE

## Anti-diabetic Efficacy Comparison of Elenolic Acid with Metformin and Liraglutide in db/db Mice

**Abstract**

Type 2 diabetes (T2D) is a fast-growing chronic disease, accompanied by pancreatic β-cell dysfunction and insulin resistance. Even though over thirty medications were approved to treat T2D in the United States, less than one in four patients with T2D treated with anti-diabetic drugs achieved the glycemic target. Thus, identifying more effective anti-diabetic drugs is still needed for improving glycemic control in patients in T2D. Recently, I found that a novel molecule, elenolic acid (EA), derived from olive and extra virgin olive oil, exhibited potent anti-diabetic and anti-obesity effects in diet-induced middle-aged obese mice. To further assess the anti-diabetic efficacy of EA, I compared the anti-diabetic potency of EA with two widely used anti-diabetic drugs, metformin and liraglutide in db/db mice. EA treatment reversed hyperglycemia in db/db mice, which was comparable to liraglutide-treated mice. However, metformin failed to improve glycemic control during the treatment period in db/db mice. The body weight gain of mice treated with EA and liraglutide was significantly less versus that of control mice ($p<0.001$), with an increase of 15.5% for EA and 23.3% for liraglutide over 4 weeks of treatment. Similarly, average food intake of EA-treated mice was 32.9% lower than that of control mice ($p<0.001$), which was comparable to the mice treated with liraglutide (26.0%). Metformin didn't significantly alter body weight gain in diabetic mice. Consistent with previous findings in diet-induced obese mice, EA treatment improved postprandial GLP-1 in response to the food intake in db/db mice, with improved glucose-stimulated insulin secretion and reduced HOMA-IR. These results suggest that EA has similar potency as liraglutide but better than metformin for blood glucose and body weight control in db/db mice.

**Key words**: Type 2 diabetes, elenolic acid, liraglutide, metformin, and db/db mice

## Introduction

Type 2 diabetes (T2D) is a fast-growing chronic disease, accompanied by insulin resistance and pancreatic β-cell dysfunction [1, 2]. Therefore, preservation of β-cell function and/or insulin sensitivity is the most therapeutic avenue for treating T2D [3]. Various classes of anti-diabetic drugs have been developed for T2D to control glycemia, including insulin (insulin analogue), biguanides, sulfonylureas, thiazolidinediones, a-glucosidase inhibitors, sodium glucose co-transporters 2 (SGLT2) inhibitors, amylin analogues, and incretin mimetics. Specifically, metformin, a biguanide, is the first line of medication typically prescribed to the newly diagnosed patient with T2D [4]. The main target of metformin is the liver where it suppresses hepatic glucose production, thereby ameliorating fasting hyperglycemia [5]. In addition to metformin, sulfonylurea drugs are also commonly prescribed medication, which bind to sulfonylurea receptors of ATP-sensitive potassium ($K_{ATP}$) channels on pancreatic β-cells to cause the closure of $K_{ATP}$ channels and thus increase insulin secretion [6]. Even though over thirty medications were approved to treat T2D in the United States [7], less than one in four patients with T2D that were treated with anti-diabetic drugs achieved the glycemic target [8]. For example, the incidence of metformin therapy failure at 5 years was 21% [9], suggesting that the glycemic response to metformin treatment is different among patients with T2D [10]. In addition, several studies showed that metformin is unable to stop the progressive decline in β-cell function and mass [11-14]. Also, it was reported that the failure rate of sulfonylurea monotherapy is 34% [9].

Incretin mimetics are one of the most efficacious anti-diabetic drugs that result in better glycemic control. Incretin-stimulated insulin release accounts for more than 50% of postprandial insulin secretion in healthy subjects [15, 16]. Glucagon-like peptide 1 (GLP-1), a well-studied incretin, is secreted from intestinal enteroendocrine L-cells after food ingestion, and plays a critical

role in maintaining glycemic homeostasis via potentiating glucose-stimulated insulin secretion (GSIS) and promoting β-cell proliferation and survival [17-19]. Thus, GLP-1-based therapy has been widely used to treat T2D. However, patients given GLP-1-based drugs, which require injection and are expensive, suffer significant side effects such as nausea and vomiting [20]. More importantly, recent studies have found that the use of incretin-based drugs raised substantial concerns of a possible increased risk of pancreatitis and C-cell hyperplasia [20-22]. Furthermore, dipeptidyl peptidase-4 (DPP-IV) is a ubiquitous enzyme involved not only in the enzymatic cleavage of numerous other peptide substances other than GLP-1, but also in non-enzymatic interactions with some proteins [23], highlighting a wide range of biological functions of DPP-IV. Therefore, indiscriminate inhibition of DDP-IV may affect a spectrum of important physiological functions of DDP-IV other than degradation of GLP-1. Given these substantial safety concerns, development of safer, cheaper, and more convenient agents that can directly promote GLP-1 secretory function of L-cells, thereby increasing insulin secretion and preserving β-cell mass, holds great potential for the treatment of T2D.

Naturally occurring agents have been shown a great potential in preventing and treating T2D in the past decades, providing a wide source for anti-diabetic drug discovery. Notably, metformin was derived from the extract of *Galega officinalis* Linn, a widely used herb to treated diabetes in 1920s [24]. Additionally, phlorizin, which was originally derived from the bark of apple tree, is the first drug discovered as the inhibitor of SGLT2 [25, 26], although is discontinued for its severe side effect in clinical use [27]. Furthermore, the first approved incretin mimetic, exendin-4 is a naturally occurring GLP-1 analog that was originally isolated from the salivary secretions of *Heloderma suspectum* [28, 29]. Recently, I found that an olive-derived compound, elenolic acid (EA), is a novel oral GLP-1 agonist with potent antihyperglycemia and anti-obesity

actions in diet-induced obese mice (DIO) (Chapters 3 and 4 of this dissertation). However, DIO mice are considered insulin resistant and glucose intolerant, and they are not overtly diabetic. In addition, the efficacy of EA as compared with currently used anti-diabetic drugs is unclear. I thus used leptin receptor-deficient (db/db) mice, a widely used T2D model with hyperglycemia, glucose intolerance, and hyperinsulinemia [30, 31], to determine whether EA exerts similar antihyperglycemic and beneficial metabolic actions, and further compare its anti-diabetic potency with liraglutide and metformin.

## Materials and Methods

### Chemicals

Elenolic acid was generated as described in Chapter 3 of this dissertation. Metformin was from Cayman Chemicals (Ann Arbor, MI); Liraglutide was from MedChem Express LLC (Monmouth Junction, NJ); insulin, methylcellulose, diprotin A, and TRI reagent were from Sigma (St. Louis, MO); High-Capacity cDNA Reverse Transcription Kit and Power SYBR™ Green PCR Master Mix were from Applied Biosystems (Grand Island, NY); primers were synthesized by Eurofins Genomics (Louisville, KY); mouse insulin ELISA kit was from Crystal Chem (Elk Grove Village, IL); glucose meter was from AgaMatrix (Salem, NH); Metabolism Multiplex Assay were from Millipore (Burlington, MA).

### Animal study

All animal studies were performed in accordance with guidelines of animal care and use committee from Virginia Tech. Five wks old male C57BLKS/J db/db and colony heterozygous mice (db/m) were purchased from The Jackson Laboratory (Bar Harbor, ME). db/m mice were used as the controls in all experiments. All mice were kept in the light (12 h light/12 h dark), temperature (23℃) and humidity (55%)-controlled room with *ad libitum* access to food and water. Following acclimation for 2 wks. Forty-two db/db mice were randomly divided into 5 groups, and 20 male db/m mice were randomly divided into two groups. The tested compounds were administered as shown in Table 1. Non-fasting blood glucose and food intake were measured weekly. Fasting blood glucose and body composition were measured at the beginning and the end of the study.

*Table 1. Treatment of db/db and db/m*

| Strain | Treatment | N | Treatment Route | Dose |
|---|---|---|---|---|
| *db/db* | Oral gavage control (OG) | 8 | Oral gavage | 2% methylcellulose |
| *db/db* | Metformin (Met) | 8 | Oral gavage | 300 mg/kg |
| *db/db* | Elenolic acid (EA) | 9 | Oral gavage | 50 mg/kg |
| *db/db* | SC injection control (SC) | 8 | SC injection | 0.9% NaCl |
| *db/db* | Liraglutide (LIG) | 9 | SC injection | 0.2 mg/kg |
| *db/m* | Oral gavage control (LOG) | 10 | Oral gavage | 2% methylcellulose |
| *db/m* | SC injection control (LSC) | 10 | SC injection | 0.9% NaCl |

**Glucose tolerance test (GTT) and insulin sensitivity test (ITT)**

For GTT, mice were fasted for 6 h and administered 1 g/kg glucose via i.p. injection, and blood glucose in tail vein blood samples was measured at time 0, 15, 30, 60, and 120 min after glucose injection using a glucose meter (AgaMatrix). For ITT, mice were fasted 6 h and then i.p. injected 0.75 U/kg insulin for db/m, and 1.5 U/kg insulin for db/db mice. Blood glucose was measured at 0, 15, 30, 60, 120, and 150 min post-injection of insulin.

**Postprandial hormone measurement**

For evaluating whether EA affects postprandial active GLP-1, GIP, and PYY release, mice fasted for 6 h and then orally administered 50 mg/kg EA, 300 mg/kg metformin, or vehicle. Afterward, mice were refed with standard chow diet for 1 h. Blood samples were drawn after fasting and refeeding and were collected into 1.5 ml tubes coated with 5 mM EDTA and 10 µM diprotin A

and centrifuged at 3,000 rpm for 15 min at 4 ºC. Plasma was obtained and stored at -80 ºC for measuring GLP-1, GIP, and PYY levels according to the manufacturer's protocol (MilliporeSigma).

**Glucose stimulated insulin secretion (GSIS)**

Mice were fasted 6 h and administered 1 g/kg glucose via i.p. injection. Blood samples were obtained before and 15 min after glucose administration. The plasma was collected and used for insulin concentration measurement (Crystal Chem).

**Hypothalamic mRNA measurements**

Hypothalamus was collected immediately after mice were euthanized. Total RNA was isolated using TRI reagent and quality and concentration of RNA were determined by NanoDrop™ 2000 spectrophotometers. RNA (500 ng) was reverse transcribed to cDNA with high-capacity cDNA reverse transcription kit following the manufacturer's protocol. Quantitative PCR was preformed using Power SYBR™ Green PCR Master Mix with GAPDH as an internal control. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method as described previously [32]. The primers of Agrp, Npy, and Pomc were listed in the Chapter 4 of this dissertation.

**Statistical analysis**

Data were analyzed by unpaired student's t-test or one-way ANOVA followed by Duncan's multiple range test using JMP 13 (SAS Institute Inc, Cary, NC). The $p < 0.05$ was considered significant difference. Values are presented as mean ± standard error of mean (SEM).

## Results

### EA had similar efficacy with liraglutide in ameliorating hyperglycemia

To evaluate whether EA also exerts anti-diabetic effects in db/db mice as observed in DIO mice and further compare its anti-diabetic efficacy relative to that of liraglutide and metformin, I measured non-fasting blood glucose (NFBG) weekly during the 4-wk treatment. At the beginning of the treatment, all groups of db/db mice displayed identical severe hyperglycemia (NFBG ≈ 450 mg/dl), while all db/m mice were euglycemic (NFBG ≈ 230 mg/dl). Notably, oral administration of EA completely reversed NFBG, which was reduced from 459 ± 51 mg/dl to 170 ± 8 mg/dl ($P< 0.01$) after only 1 wk of treatment, and mice remained euglycemic with NFBG levels at about 200 mg/kg after 4 wk of treatment ($P< 0.01$), which were comparable to those of db/m mice. However, NFBG levels of untreated db/db mice were still severely hyperglycemic (465 ± 50 mg/dl) at the end of the treatment. Liraglutide treatment reduced NFBG levels from 454 ± 55 mg/dl to 344 ± 28 mg/dl ($P< 0.01$) after 1 wk of treatment, which was further decreased to 318 ± 44 mg/dl ($P<0.01$) following 4 wk, whereas NFBG levels in its control mice were 483 ± 42 mg/dl. Further analysis showed that mice treated with EA had significantly lower NFBG levels as compared with those in liraglutide-treated mice at the end of 1wk, 2wk, and 4wk of the treatment ($P < 0.05$), suggesting that at the tested dose, EA is more potent than liraglutide in lowering non-fasting hyperglycemia. Interestingly, metformin didn’t significantly change blood glucose levels during the 4-wk treatment period. Consistently, the fasting blood glucose (FBG) of EA-treated mice was 179 ± 11 mg/dl at the end of the study, which was significantly lower as compared with that control mice (346 ± 49 mg/dl) and that in metformin-treated mice (300 ± 54 mg/dl) (Fig. 1B). In addition, the FBG of liraglutide-treated mice (196 ± 8 mg/dl) was significantly lower relative to that in its control mice (340 ± 47 mg/dl). Impressively, EA was also more effective in lowering fasting

hyperglycemia as compared to liraglutide, but the difference between the two groups was not statistically significant.

Glucose tolerance test showed that both EA-treated and liraglutide-treated mice exhibited better glucose tolerance as compared to their respective controls (Fig. 1C and D), but AUC from EA-treated mice were significantly higher in comparison to that of liraglutide-treated mice ($P< 0.05$). Metformin treatment had no effect on glucose tolerance. In consistent with glucose tolerance test, control db/db mice had no significant insulin response to glucose stimuli, while metformin, EA, and liraglutide-treated mice showed a robust increase in plasma insulin in response to glucose challenge (Fig. 1E), suggesting that metformin, EA, and liraglutide administration potentiated GSIS in db/db mice. The HOMA-IR was calculated for assessing the insulin sensitivity of the mice, and showed that EA and liraglutide-treated mice, but not metformin-treated mice, had a significantly lower HOMA-IR value relative to their controls, suggesting EA and liraglutide administration might also improve the insulin sensitivity in db/db mice (Fig. 1F). There was no significant difference of HOMA-IR between EA and liraglutide treated mice. However, neither EA-treated mice nor liraglutide-treated mice improved insulin sensitivity relative to their control mice from the ITT tests (Fig. 1G and H).

**EA and liraglutide reduced the weight gain**

Previous study showed that oral administration of EA significantly reduced the body weight in DIO mice (Chapter 4). To determine if oral administration of EA reduces the weight gain in db/db mice, I measured the body weight through the time course of the treatment. As expected, the body weights of db/db mice were significantly higher than those of db/m mice (Fig. 2A and B). The initial body weight before the treatment was around 33g in db/db mice and 25 g

in db/m mice, After 4 wks of treatment, the average body weight of EA-treated db/db mice (39.3 ± 1.2 g) was lower than that of vehicle-treated db/db mice (45.9 ± 1.4 g) ($P< 0.05$), and also lower than that of liraglutide-treated mice (43.9 ± 1.3 g, $P< 0.05$). However, there was no significant difference in body weight between metformin-treated and its control mice, or liraglutide-treated and its control mice. At the end of 4 wks, the body weight of EA-treated mice was increased by 15.5%, which was more than 50% less as compared to the control mice ($P < 0.05$), whereas the body weight of metformin-treated mice was similar to that of the control mice (Fig. 2C). Body weight of liraglutide-treated db/db mice was increased by 23.3% at the end of the treatment, which was 30% less than that of the control mice ($P<0.05$). However, no treatment significantly altered the percentage of fat mass (Fig. 2D). In contrast, mice treated with EA or liraglutide had a lower fat mass gain as compared with their relative controls (Fig. 2E), suggesting that EA and liraglutide mitigated the obesity development in db/db mice. EA-treated mice had greatly lower average food intake (3.8 ± 0.2 g/day/mouse, $P<0.001$) as compared to its control mice (5.7 ± 0.1 g/day/mouse), which was 32.9% lower than its control, confirming a strong anorexigenic effect of EA. Metformin treatment also moderately but significantly reduced food intake (5.0 ± 0.1 g/day/mouse, $P<0.01$) as compared to the control db/db mice (Fig. 2F). As expected, liraglutide -treated mice had a lower food intake (4.2 ± 0.2 g/day/mouse, $P <0.001$) as compared to its control mice (5.6 ± 0.2 g/day/mouse), which was 26.0% lower than its control consistent with its well-known anorexigenic action. While EA-treated mice had a lower food intake as compared to liraglutide-treated mice, the difference was not statistically significant.

**EA increased postprandial GLP-1 response**

Next aim was to determine whether EA or metformin administration increases postprandial concentrations of gut hormones, GLP-1, peptide tyrosine tyrosine (PYY), and gastric inhibitory polypeptide (GIP), mice after 3-wk treatment were fasted for 6 h and then were given EA (50mg/kg), metformin (300 mg/kg), or vehicle followed by 1 h of refeeding. Blood samples were drawn and measured using a multiplex magnetic metabolic panel kit. Fasting GLP-1 concentration levels were comparable between the control and metformin and EA-treated mice (Fig.3A). While EA administration had a significant higher postprandial GLP-1 concentration (131.8 ± 27.1 pg/ml, $P < 0.05$) as compared to that of the fasting condition (69.9 ± 19.8 pg/ml), postprandial GLP-1 concentration was comparable to its control. However, neither fasting GLP-1 nor postprandial GLP-1 concentration was significantly different between treatments in db/db mice. Fasting GIP concentration was significantly lower in metformin-treated mice as compared to that of its control (Fig.3B). However, the fasting GIP concentration was comparable between EA-treated mice and its control. While postprandial GIP concentrations from each treatment were significantly higher relative to their fasting GIP level, postprandial GIP had no significant difference between each treatment. Similarly, fasting PYY concentration of metformin-treated mice was significantly lower as compared to that of its control, but no difference of fasting PYY between EA-treated mice and the control (Fig.3C). In addition, postprandial PYY concentrations from each treatment were significantly higher than that of their relative fasting PYY, levels, but postprandial PYY levels in the circulation were no significant different between the treatment groups.

PYY and GLP-1 were reported to regulate food intake through activating their respective receptors in the hypothalamic arcuate nucleus (ARC) to regulate the production of neuropeptides, of which neuropeptide Y (Npy), agouti-related peptide (Agrp), and proopiomelanocortin (Pomc) are usually used to assess the effect of gut hormones on hypothalamus [33, 34]. To evaluate the

effect of EA on hypothalamic neuropeptide expression levels in db/db mice, I harvested the postprandial hypothalamus, isolated RNA, and measured the mRNA expression of Npy, Agrp, and Pomc (Fig. 3D). There was no significant difference in Npy mRNA abundance between EA, metformin, or liraglutide treatment group and their respective control mice. Consistent with the observation in DIO mice, mice treated with EA had significantly lower hypothalamic orexigenic Agrp expression level as compared with its control db/db mice, while liraglutide-treated mice had higher Pomc mRNA levels as compared to its control diabetic mice.

**Discussion**

It is well-established that GLP-1 plays a vital role in maintaining glycemic control via improving β-cell function [35]. Thus, several GLP-1-based drugs have been developed to treat T2D. In this study, I present results using an overt T2D mouse models that EA, a naturally occurring GLP-1 agonist, indeed has novel anti-diabetic effects with potent blood glucose-lowering action. It is worth noting that EA was comparably efficacious with liraglutide in blood glycemic control but was better than that of metformin in overt diabetic db/db mice. In line with our previous study in DIO mice, EA also suppressed food intake and reduced body weight gain in db/db mice, which are consistent with the established metabolic effects of GLP-1. Therefore, EA may be a naturally occurring anti-diabetic compound with pronounced effects on glycemic and body weight control.

Metformin is a first-line anti-diabetic drug prescribed to the newly diagnosed patients with T2D. The dose of metformin used in our study was 300 mg/kg, which is equivalent to 1,700 mg/day for a 70 kg person based on a widely used conversion methods from human to mouse [36]. Clinically, the initial prescribed dose of metformin is 500 mg/day but it is titrated up to 2,000 mg/day once a patient fails to achieve the target the desired glycemic target [37]. However, in this study, metformin (300 mg/kg) administration failed to improve glycemic control in db/db mice. This failure of lowering blood glucose by this dose of metformin treatment should not be due to its effectiveness, as oral metformin administration significantly reduced food intake but had no effect on body weight or fat mass in db/db mice, which is consistent with the observation from previous studies [38, 39]. It is conceivable to assume that the persistent hyperglycemia in this model might have reached to the magnitude that monotherapy with metformin alone is incapable of providing effective glycemic control, which often occurs in T2D patients. The db/db mice are

widely used animal model of human T2D characterized by obesity, hyperglycemia, insulin resistance accompanied with dysfunction of the islets [40]. It is well known that metformin suppresses hepatic glucose production, decreases intestinal glucose absorption, and increases peripheral glucose uptake [41]. While metformin may be effective in improving insulin sensitivity, it may not be able to preserve pancreatic β-cell mass and function over time. Given that db/db mice used in this study developed the severe hyperglycemia accompanied with significantly reduced β-cell function, metformin at the dose used may be insufficient in improving the overt diabetes, suggesting that metformin might not reverse the hyperglycemia that induced by insufficiency of insulin. Indeed, it has been found that metformin lacks the effects on pancreatic β-cell function *in vivo* [42, 43], even though metformin potentiates GSIS from cultured mouse islets [44], and protects pancreatic β-cell lines from glucolipotoxicity and lipotoxicity [45, 46].

Oral formulation of GLP-1 agonist for treating T2D is still limited but needed, as it is convenient and simply administered for patients in T2D. So far, there is only one oral GLP-1 receptor agonist (GLP-1RA), Rybelsus, from Novo Nordisk that has been recently approved by FDA, whereas pharmaceutical industry has already noticed the value of oral GLP-1RAs, and another oral GLP-1RA, OWL833 [47], from Eli Lilly, is going to enter Phase 1 clinical development [48]. Liraglutide is an injectable peptide and shares 97% sequence identity to human GLP-1 [49] and resistant from the degradation by DPP-IV via the amino acid addition, acylation, and substitution [50]. In addition, the C16 fatty acid, linking with lysine (at position 26) in the liraglutide, largely increases the half-life of liraglutide to 20 h in the circulation [51]. And thus, patients only need to receive the injection of liraglutide once daily. Even though the frequency of liraglutide injection is lower than other insulin-based drugs, patients still fear the injection pain, which might jeopardize the efficacy of treatment. While GLP-1 analogues, also known as GLP-

1RAs, are an effective class of anti-diabetic drugs, several of them induced a reduction of postprandial GLP-1 concentrations [52, 53], suggesting the GLP-1 analogues might impact the intact GLP-1 secretion. In addition, GLP-1RAs are developed for clinical use with a structural heterogeneity to the native GLP-1, which may result in the difference in GLP-1R activation or preferential access to GLP-1Rs of central nervous systems and lead to the different efficacy on glycemic and body weight control [54]. As shown in Chapter 4, EA is an oral GLP-1 agonist, inducing a significant postprandial GLP-1 secretion with the dose of 50 mg/kg in mice. As compared to the injectable GLP-1RA, liraglutide, diabetic mice received EA achieved similar glycemic control, and even superior to liraglutide in controlling body weight gain in db/db mice, suggesting that EA may be a potent oral GLP-1 agonist *in vivo*.

Native GLP-1 directly binds to the GLP-1R on the hypothalamic POMC neuron to exert its regulatory action in food intake, and indirectly inhibits the activity of Npy/Agrp neuron via binding the gamma-aminobutyric acid (GABA) receptor [55]. However, in present study, oral EA administration induced a reduction of the expression of hypothalamic Agrp in db/db mice but had no significant effects on the expression of hypothalamic Npy and Pomc, which is consistent with the observation in DIO mice (Chapter 4). In addition, liraglutide increased the expression of anorexigenic neuropeptide, Pomc, whereas it didn't affect the expression of Npy/Agrp, which might result from the indirect binding of liraglutide to the GABA-dependent signaling on the neurons of Npy and Agrp, as there is no GLP-1R in the neurons of Npy and Agrp [56, 57]. As aforementioned, the structural heterogeneity of liraglutide to native GLP-1 might lead to their preferential access to hypothalamic GLP-1R or GABA receptor and leads to the differential hypothalamic actions. While EA administration suppresses the expression of hypothalamic Agrp, the reduction of food intake is comparable to that of liraglutide, suggesting that another mechanism

exists that might partially mediated EA suppression of food intake. In that regard, EA could inhibit the secretion of orexigenic hormone, ghrelin. It was revealed previously that activation of $G\alpha_q$-dependent G-protein coupled receptor (GPCR) leads to the suppression of ghrelin secretion [58]. As shown in Chapter 3, EA activates the $G\alpha_q$-mediated signaling pathway, albeit in L-cells, it is possible that EA may inhibit ghrelin secretion, which needs further investigation.

In summary, the findings showed that the anti-diabetic efficacy of EA is comparable to that of the widely used GLP-1 agonist, liraglutide. Oral EA administration improved the postprandial GLP-1 in response to the food intake, which may be at least in part responsible for suppressed food intake and body weight gain, given that GLP-1 is involved in the central regulation of food intake and suppression of stomach emptying rate. These findings are in line with the results from studying DIO mice (Chapter 4). Collectively, I have identified for the first time, as to the best of our knowledge, that EA could be a dual-action compound that exerts anti-diabetic effects via activation of GLP-1 mediated metabolic pathway and suppression of hepatic gluconeogenesis, leading to effective control on food intake, body weight gain, and glycemic control in T2D mice.

## Figures

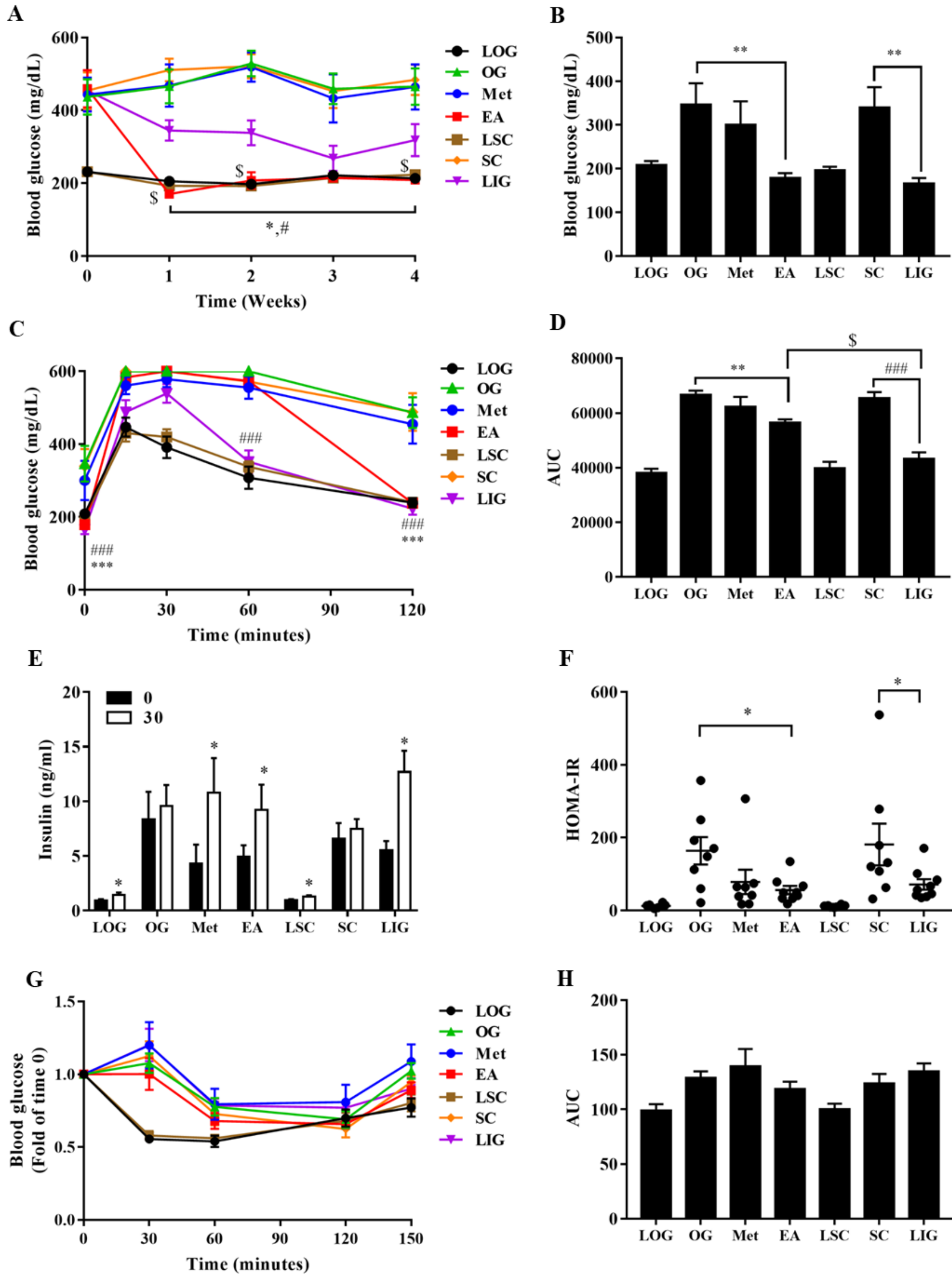

**Figure 1. EA had similar efficacy with liraglutide in ameliorating hyperglycemia.** Non-fasting blood glucose (A) was measured every week during the treatment. (B) Fasting blood glucose was measured at the beginning and at the end of the study. (C) 6 h-fasted mice were given 1 g/kg glucose via i.p injection and blood glucose were measured at 0, 15, 30, 60, 90, and 120min after glucose administration. The corresponding area under the curve was shown in D. (E) Blood samples were obtained after 6 h-fasting and after 30 min of glucose injection. Insulin concentration was measured using ELISA kit. * $P< 0.05$ vs. time 0. (F) HOMA-IR was calculated based on fasting blood glucose and fasting insulin concentration. (G) 6 h-fasted mice were given 0.75 U/kg insulin (db/m) or 1.5 U/kg insulin (db/db) via i.p injection. The blood glucose was measured after 0, 30, 60, 90, 120, and 150 min after insulin injection. The corresponding area under the curve was shown in H. Data were presented as means ± SEM, * $P < 0.05$ vs. OG; # $P< 0.05$ vs. SC. $ $P < 0.05$ vs. LIG.

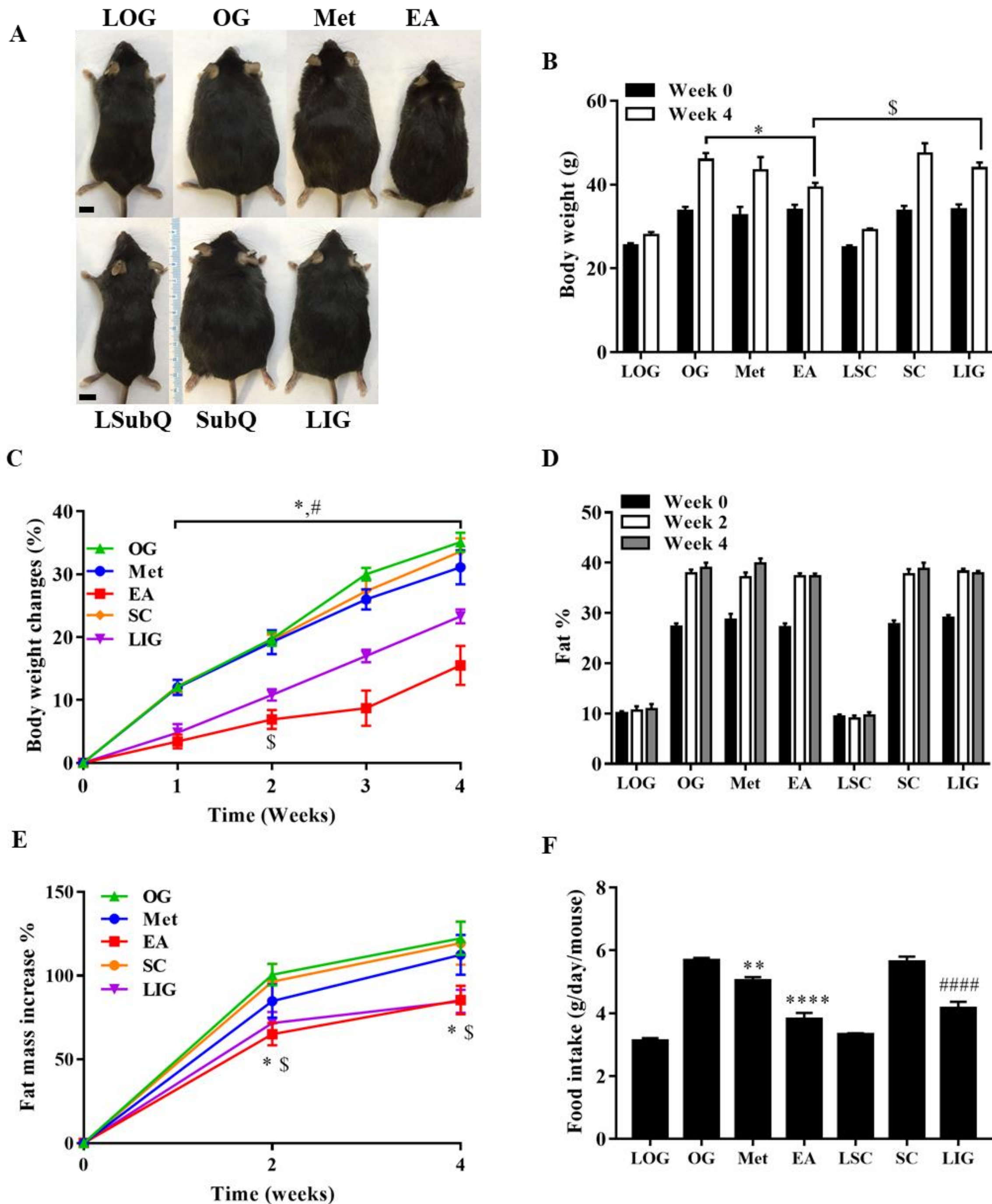


**Figure 2. EA and liraglutide reduced the weight gain.** (A) Picture of one mouse from each treatment was taken at the end of the treatment. Scale bar = 1cm. Body weight (B) and changes of body weight (C) were recorded. Fat percentage (D) and fat mass increase (E) were measured through the study. (F) The average food intake during the study was calculated. Data were presented as means ± SEM, * P < 0.05 vs. OG; # P< 0.05 vs. SC; $ P < 0.05 vs. LIG.

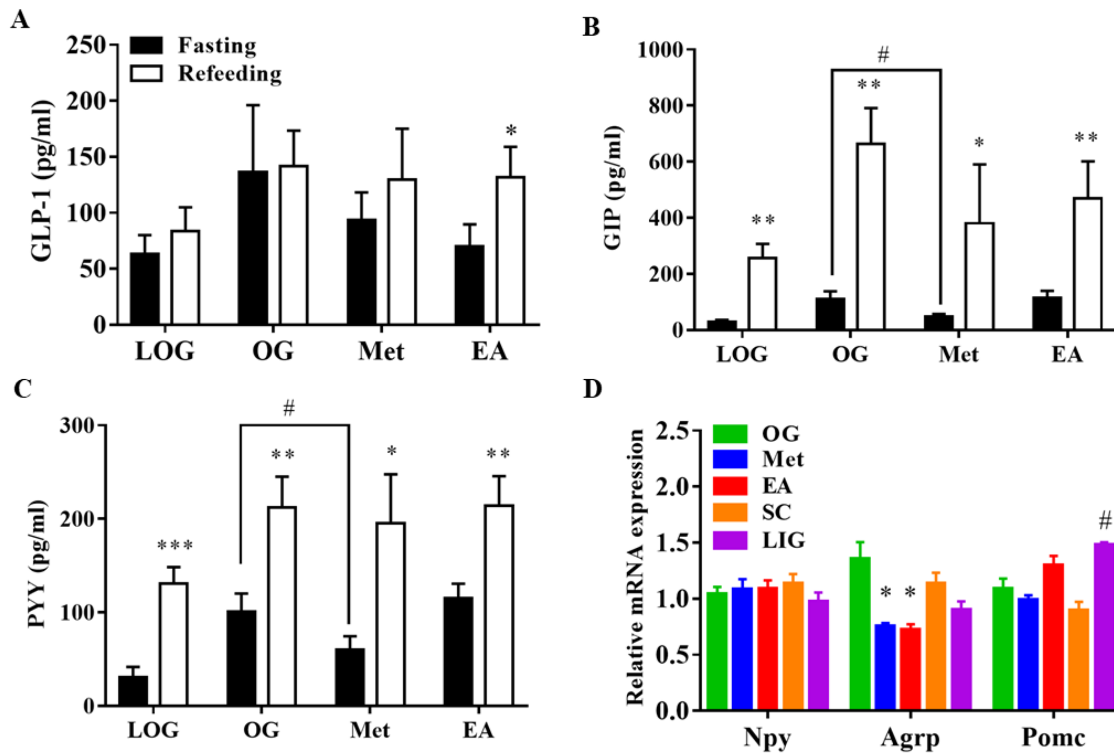


**Figure 3. EA increased postprandial GLP-1 response.** Blood samples were drawn after 6 h-fasting and 1 h refeeding. Plasma were used for active GLP-1 (A), GIP (B), and PYY (C) measurement. Data were presented as means ± SEM, * P < 0.05 vs. fasting. Hypothalamus was collected from mice after 1h refeeding. mRNA was isolated for hypothalamic neuropeptide, Npy, Agrp, and Pomc analysis (D). Data were presented as means ± 95% CI, * P < 0.05 vs. OG; # P< 0.05 vs. SC.

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# CHAPTER SIX

## Conclusion

In this dissertation, I provide strong evidence from studying mouse L-cells, diet-induced obese (DIO) mice, and db/db mice that the olive-derived small molecule elenolic acid (EA) is a dual action, anti-diabetic agent with potent blood glucose lowering and anti-obesity effects. From the *in vitro* study, I found that EA stimulated GLP-1 secretion via activation of the $G\alpha_q$-PLC-$IP_3$-$Ca^{2+}$ pathway. Therefore, it is attempting to speculate that EA might exert anti-diabetic potential due to its stimulatory effect on GLP-1 secretion. Indeed, EA showed anti-diabetic and anti-obesity action in DIO mice via lowering blood glucose and controlling body weight, as a result of promoting GLP-1 secretion, and thereby the delay gastric emptying rate and reduced food intake, as well as body weight loss. Consistently, EA exhibited anti-diabetic and anti-obesity effects in db/db mice. Notably, the efficacy of EA was better than that of metformin, but comparable to that of the widely used GLP-1 agonist, liraglutide. Oral EA administration improved postprandial GLP-1 in response to food intake, which may be at least in part responsible for the decreased food intake and obesity, given that GLP-1 is involved in central regulation of food intake and suppression of stomach emptying rate. These findings are in line with the results from studying DIO mice. Collectively, I identified for the first time, as to the best of our knowledge, that EA could be a dual action compound that exerts anti-diabetic effects via activation of GLP-1 mediated metabolic pathway and suppression of hepatic gluconeogenesis, leading to effective control on food intake, body weight gain, and glycemic control.

### Directions for future research

In this dissertation, I demonstrated that EA is a potent anti-diabetic and anti-obesity small molecular agent in two obese and diabetic mouse models for T2D. However, more studies are still needed to investigate the cellular and molecular mechanism that involved in the anti-diabetic effects of EA. The following aspects that need to be investigated.

1) The exact cellular and molecular mechanism by which EA activates GLP-1 secretion is still unclear. While EA activated Gαq signaling to induce GLP-1 secretion, how EA activates $G\alpha_q$ is still unclear. As $G\alpha_q$ is coupled to a membrane receptor, it is likely that EA binds with a receptor and then induce the intracellular signaling. Thus, it is necessary to identify the receptor that activated by EA.

2) How EA regulates hepatic gluconeogenesis. Hepatic gluconeogenesis is precisely regulated by insulin and glucagon. Given GLP-1 stimulates insulin secretion and suppresses glucagon secretion, the reduced hepatic gluconeogenesis might result from the increment of GLP-1 concentration *in vivo*. However, *in vitro* study showed that EA inhibited glucose production in the human liver cell line (HepG2), suggesting EA might function on hepatocytes directly.

3) It is unclear the dose-dependent action. I have used 50 mg/kg as our experimental dose in all *in vivo* studies. Thus, the dose-dependent action of EA is largely unknown. As shown that EA dose-dependently induced GLP-1 secretion *in vitro*, it is likely that the anti-diabetic action of EA *in vivo* depending on its doses.

4) The bioavailability of EA is unclear. Most dietary polyphenols have an extremely low (< 5 μM) concentration in the circulation. Given EA might have the anti-diabetic effects on other tissues, for example, liver, investigation of EA bioavailability would be of the vital importance to understand the mechanism of EA's action *in vivo*.

5) The potential toxicity needs to be carefully examined. As EA is novel anti-diabetic agent that derived from olive and extra virgin olive oil, its toxicity has not been fully studied. It is necessary to examine the toxicity for further study.