THE BIOACTIVE EFFECTS OF CHICORIC ACID AS A FUNCTIONAL FOOD INGREDIENT

THE BIOACTIVE EFFECTS OF CHICORIC ACID AS A FUNCTIONAL FOOD INGREDIENT

A Dissertation Presented

by

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DEDICATIONS

Dedicated to my husband, my parents, my twin sister,

and parents-in-law.

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ABSTRACT

THE BIOACTIVE EFFECTS OF CHICORIC ACID AS

A FUNCTIONAL FOOD INGREDIENT

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Chicoric acid, a hydroxycinnamic acid, has been reported to possess a variety of health benefits, including antivirus, anti-oxidation, anti-inflammation, obesity prevention and neuroprotection effects. However, its role on aging and glucose homeostasis are largely unknown. Therefore, the bioactive effects of chicoric acid on aging and glucose homeostasis were determined using *Caenorhabditis elegans* (*C. elegans*) and C2C12 myotubes, respectively. Our study showed that chicoric acid (25 and 50 μM) significantly extended the lifespan of *C. elegans* and increased median survival rates. The declines of pumping rate and locomotive activity, two indicators of aging, were delayed by chicoric acid. Chicoric acid also enhanced resistance to oxidative stress compared to the control. These effects were in part via AAK-2 (a homolog of AMP-activated protein kinase) and

SKN-1 (a homolog of nuclear factor erythroid 2-related factor 2). Furthermore, chicoric acid significantly enhanced glucose uptake and the phosphorylation of protein kinase B (Akt) as an indicator of insulin signaling pathway independent of insulin in C2C12 myotubes. These effects were dependent of the phosphorylation of 5'-AMP-activated protein kinase α (AMPKα), via increasing the AMP/ATP ratio in C2C12 myotubes. Our study may suggest the potential of chicoric acid to be used as an anti-aging and anti-diabetic bioactive compound.

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CHAPTER 1

INTRODUCTION

Chicoric acid is a hydroxycinnamic acid presents in the roots of a large number of plants (63 genera and species), including many plants grown in the Mediterranean area (*1*), and many of these plants have been consumed traditionally as food ingredients and alternative medicines for some time (*2*, *3*). Because chicoric acid is found in especially high amounts in chicory (*Cichorium intybus*), purple coneflower (*Echinacea purpurea*), and basil, the acid is often used as a marker for the quality check of herbal products from these plants (*4*). More recently, an increasing number of publications have reported the beneficial effects of chicoric acid in cell culture and animal studies. However, its role on aging and glucose homeostasis are largely unknown.

Aging is defined as a progressive decline of intrinsic physiological functions, resulting in an increased risk of death (*5*). Considering that aging is one of the key risk factors associated with many chronic diseases, slowing aging is of great importance to prevent age-associated diseases and improve the health span of life (*6*). A previous study reported that chicoric acid extend lifespan in *Caenorhabditis elegans* (*7*), its effects on aging-related phenotypes and its underlying mechanisms, however, remain to be determined.

Type 2 diabetes mellitus (T2DM) has been recognized as a major public health challenge throughout the world in recent decades (*8*, *9*). Impaired glucose uptake in muscle tissues is the primary associated with hyperglycemia in T2DM (*10*). Previous in vitro studies reported that chicoric acid ameliorated insulin resistance in human hepatoma cells (*11*). However, the effects of chicoric acid on muscle cells and its underlying

mechanism are still unclear. Here, C2C12 myotubes, derived from murine skeletal muscle cells and a C57BL/6J mice model were used to determine the role of chicoric acid on glucose homeostasis and its underlying mechanisms were determined.

CHAPTER 2

LITERATURE REVIEW

2.1 Chicoric acid

Chicoric acid, a hydroxycinnamic acid that is a member of the phenylpropanoid family, contains two caffeoyl units (*2*). Chicoric acid mainly presents in two forms: first, the most abundant natural form of chicoric acid is L-chicoric acid (Figure 1); second, the acid presents in the stereoisomer meso-chicoric acid (i.e., dicaffeoyl-meso-tartaric acid or di-E-caffeoyl-(2*R*-3*S*)-(-)-tartaric acid) (*1*).

Chicoric acid is present in the roots of a large number of plants (63 genera and species), including many plants grown in the Mediterranean area (*1*), and many of these plants have been consumed as alternative medicines or food supplements for some time (*2, 3*). Because chicoric acid is found in especially high amounts in chicory (*Cichorium intybus*), purple coneflower (*Echinacea purpurea*), and basil, the acid is often used as a marker for the quality check of herbal products from these plants (*4*). The roots of chicory and purple coneflower are usually baked, grounded and used as a coffee substitute in Europe (*12*). In Turkey, an herbal tea made from chicory has been used historically for the treatment of diabetes, epilepsy, hemorrhoids, inflammation and digestive disorders (*13*). The plant roots containing chicoric acid have been used in Asian traditional medicine as a tonic for curing infectious diseases, inflammatory diseases, eye diseases, and nerves injuries (*13, 14*). Commercial products made from purple coneflower are currently popular alternative medicines widely used in North America for cold and flu prevention (*15*). More recently, an increasing number of publications have reported the beneficial effects of chicoric acid in cell culture and animal studies. This

review summarizes these health benefit studies and the underlying mechanisms of chicoric acid.

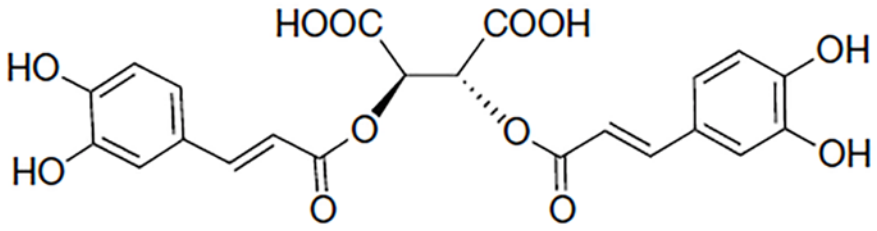


Figure 2.1. Molecular structure of L-chicoric acid. The most abundant natural form of chicoric acid is L-chicoric acid, i.e., (-)-chicoric acid, 2,3-dicaffeoyl-L-tartaric acid, 2,3-O-dicaffeoyltartaric acid, 2*R*,3*R*-O-dicaffeoyltartaric acid, or di-E-caffeoyl-(2*R*-3*R*)-(-)-tartaric acid.

2.2 The Biological effects of chicoric acid

The main benefits of chicoric acid include antivirus, anti-inflammation, glucose and lipid homeostasis, neuroprotection, and anti-oxidation effects. Biological activities and related mechanisms of chicoric acid from in vitro and in vivo studies are summarized in Tables 1 and 2.

2.2.1 Anti-viral effects of chicoric acid

The first reported bioactive effect of chicoric acid is its ability to inhibit infection with human immunodeficiency virus 1 (HIV-1) (*16*). Several studies reported that chicoric acid inhibits infection with HIV-1 by deactivating HIV-1 integrase (*16-23*). HIV-1 integrase is a multidomain enzyme required for the integration of viral DNA into the host genome, a critical step in viral replication (*24*). The inhibition of HIV-1 integration by chicoric acid results in the stopping virus replication, leading to increased T-lymphoblastoid cell viability (*17-19, 21, 22*). It has been further suggested that chicoric

acid decreases integrase binding site activity, including: the downregulation of HIV 3'-end processing products (*16*), the occupation of HIV-1 integrase catalytic core (*20*), the chelation of integrase divalent cations (*17*), the increase of long terminal repeat circle formation (*21*), and the inhibition of integrase-mediated catalysis (*18*). Hu et al. (*25*) reported that the mutation of HIV integrase might result in the blocking of chicoric acid specific binding sites and King et al. (*26*) further reported that the mutation on glycine to serine at position 140 (G140S) of HIV integrase reduced chicoric acid's effects on HIV infection, suggesting that the integrase G140S might be a target site of chicoric acid. There are a few studies that are using new computational techniques including molecular docking and quantitative structure-activity relationship (QSAR) analysis to identify the binding sites of chicoric acid with HIV-1 integrase (*25, 27, 28*). Chicoric acid binds HIV-1 integrase at its two arms, including the s-cis/s-cis isomer and s-cis/s-trans isomer arrangements (*27*). Based on the observation that the s-cis/s-cis isomer exhibits the most stable binding, this site was suggested to be the target of chicoric acid to inhibit HIV integrase. Another study using QSAR analysis indicated that the poly aromatic rings of chicoric acid are central linkers in binding to HIV-1 integrase (*28*). In addition to integrase, reverse transcriptase is another potential target of chicoric acid (*23, 29*), as chicoric acid downregulates reverse transcriptase of HIV-1 through the inhibition of the trans-activator of transcription, an important protein promoting HIV-1 reverse transcription (*29*).

Although chicoric acid may be a potential treatment for HIV, there are several limitations of using naturally occurring chicoric acid as a treatment, such as poor stability and limited cell permeability due to diacid moiety (*30*). To overcome these limitations,

chicoric acid analogs have been introduced, such as an analog of a decarboxyl compound, while extending a caffeoyl group on 3,4,5-trihydroxycinnamoyl sidechains, which showed improved stability and cell bioavailability (*30-32*). This analog also exhibited the high potency against HIV integrase infectivity (*30-32*).

2.2.2 Chicoric acid and inflammatory responses

Chicoric acid has been found to ameliorate inflammation induced by lipopolysaccharides (LPS) in both cell culture and mice. Reduced inflammation was associated with down-regulations of nuclear factor κB (NF-κB) and tumor necrosis factor α (TNF-α) (*33-36*), which are two major regulators of inflammation responses (*37-39*). Several other pro-inflammatory factors – including nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β), IL-12 and IL-18 have been also reported to be downregulated by chicoric acid (*33, 35, 40-43*). However, two relevant studies showed results inconsistent with the above. Matthias et al. (*44*) reported that LPS inhibited NF-κB expression, which was reversed by chicoric acid treatments in Jurkat E6.1 leukaemic T-cell lymphoblasts. The other reported that echinacea extracts (containing chicoric acid) upregulated LPS-induced TNF-α in rat alveolar macrophages (*45*). Inconsistencies may be due to dosage and types of LPS used (i.e., 0.1 vs. 1 μg/ml) (*34, 43, 46-48*) and/or other components present in treatments (*36, 49*). Overall, since the pro-inflammatory factors above are related to the occurrence of many chronic diseases, most research suggests chicoric acid may be considered a preventive tool for inflammation-associated diseases (*33, 35, 40-43*), however, further evaluation is needed.

2.2.3 Chicoric acid and glucose metabolism

Chicoric acid has been reported to promote glucose uptake in muscle cells and hepatocytes, through activating the insulin receptor substrate/ phosphoinositide 3-kinase /protein kinase B (Akt) pathway (*11, 50, 51*). Zhu et al.(*51, 52*) suggested that chicoric acid activated Akt via the AMP-activated protein kinase α (AMPKα)-dependent mechanism, which is a master regulator for energy homeostasis and also plays a key role in glucose metabolism. However, the mechanism underlying how chicoric acid induces AMPKα activation is not known currently. In addition to AMPKα, glucosidase, a digestive enzyme for carbohydrates, was reported to be suppressed by chicory (*53*), which can contribute to reduced glucose levels *(54, 55).* Another enzyme, protein tyrosine phosphatase 1B (PTP1B) negatively regulates the insulin signaling pathway by inhibiting the activity of insulin receptor kinase (*56*). Two studies with molecular docking showed the molecular interactions between the allosteric site of PTP1B and chicoric acid, which suggests chicoric acid might inhibit PTP1B and further activate the insulin signaling pathway (*57, 58*).

In in vivo studies, chicoric acid (or leafy extracts of echinacea or basil) reduced streptozotocin induced-hyperglycemia in mice (*50, 52, 59, 60*). In these studies, the protection of pancreatic β-cells by chicoric acid was attributed to its regulation of apoptosis related-genes, including c-Jun N-terminal kinase (JNK), B-cell lymphoma 2-associated X/B-cell lymphoma 2 (Bax/Bcl-2) ratio, and pancreatic duodenal homeobox 1 (PDX-1) (*61*). In addition, as stated in the previous section, chicoric acid downregulates several pro-inflammatory cytokines and mediators and these metabolic inflammatory

cytokines are related to the impairment of glucose homeostasis (*62*), indicating that chicoric acid might improve glucose homeostasis via regulating inflammatory responses, including those associated with regulators COX-2, mitogen-activated protein kinase (MAPK), cAMP response element binding protein (CREB) and NF-κB (*51, 52*),

2.2.4 Chicoric acid and lipid metabolism & liver function

Chicoric acid has been found to reduce high-fat-diet-induced weight gain in mice (*63, 64*). This was in part via the inhibition of peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα), two critical transcription factors in adipocyte differentiation and lipid accumulation, while increasing the secretion of adiponectin (*64*). Similarly, a few studies suggested that chicoric acid protected the liver from high-fat- or alcohol-induced fat accumulation and hepatic steatosis (*63-65*). The hepatoprotective effects of chicoric acid were suggested to be due in part to a decrease in the Bax/Bcl-2 ratio and the inhibition of fatty acid synthase (FAS) and pro-inflammatory cytokines, including TNF-α, IL-6, COX-2 and JNK in the liver (*63, 64*).

2.2.5 Chicoric acid and brain function

There is a report that chicoric acid treatment ameliorated restraint stress-induced behavioral despair and depression in mice (*66*). This neuro-protective effects of chicoric acid may result from its upregulation of neurotransmitters, including noradrenaline, dopamine, and 5-hydroxy tryptamine in the whole brain region of mice (*66*). Since restraint stress is an important factor contributing to disorders and behavioral changes (such as depression, memory loss, anxiety, and learning disability), the possible

alleviation of restraint stress by chicoric acid suggests the potential application of chicoric acid for the above-mentioned brain disorders (*66*). Others reported that chicoric acid protected neurons from memory impairment, amyloidogenesis, and hippocampus shrinkage induced by LPS and D-galactose in mice (*33, 34, 67*). They also found that chicoric acid inhibited the expression of amyloid β and its downstream enzyme, neuronal β-secretase 1, both of which are known factors contributing to the disruption of neural connectivity and neuronal death (*68*). Meanwhile, chicoric acid upregulated brain-derived neurotrophic factor, which is a canonical nerve growth factor supporting the survival of existing neurons and promoting the growth of new neurons and synapses (*69*). Although limited, these findings suggest the potential protective activity of chicoric acid on controlling the pathogenesis of neuro-degenerative diseases.

2.2.6 Anti-oxidative stress effects of chicoric acid

Oxidative stress is defined as the imbalance between the generation of reactive oxygen species (ROS) and a physical ability of detoxification or damage restoration associated with ROS (*70*). ROS could act as cellular massagers but cause physical damages through disruptions of normal cell signaling pathways (*71*). Chicoric acid has been found to have a high oxygen radical scavenging capacity, reducing the ROS level and protecting cells from free radical-induced cytotoxicity (*34, 53, 72-75*). Moreover, chicoric acid increased the generation of anti-oxidative enzymes that contribute to the reduction of ROS levels, i.e., glutathione, glutathione peroxidase, superoxide dismutase, chloramphenicol acetyl transferase, heme oxygenase and NAD(P)H dehydrogenase in various cells (*15, 21, 25, 43, 49, 61, 62, 71, 76, 77*). The underlying mechanism of the

antioxidative effects of chicoric acid is attributed to the enhanced nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf-2) and the level of peroxisome proliferator-activated receptor-γ coactivator α (PGC-1α) (*34, 73, 75*). The activation of Nrf-2 and PGC-1α inhibit ROS-induced cytotoxicity by upregulation of antioxidant response-related genes and promotion of the mitochondrial antioxidant defense system, respectively (*78, 79*). Since oxidative stress is closely related to the development of certain cancers and chronic diseases, all these findings suggest the potential future application of chicoric acid for oxidative stress-associated diseases.

2.2.7 Other bioactivities of chicoric acid

Chicoric acid at relatively higher concentrations (105-315 μM) has been reported to inhibit cancer cell growth via inhibiting cell proliferation, promoting cell apoptosis, and deactivating telomerase through upregulation of DNA fragmentation, cleaved caspase-9, and cleaved poly (ADP-ribose) polymerase (*80*).

Another study suggested that chicoric acid ameliorated ultraviolet A irradiation-induced dermal fibroblast senescence by the inhibition of matrix metalloproteinase-3 activity (*81*). As dermal fibroblast senescence is a hallmark of intrinsic and ultraviolet-mediated aging (*77*), this indicates the potential role of chicoric acid in aging.

Table 2.1 In vitro effects of chicoric acid in the treatment of various disorders.

Disorders	Models	Dose (μM)	Duration (hrs)	Effects	Suggested mechanisms	References
Anti-viral effects	H9 & MT-2 human T-lymphoblastoid	1.05-10.5	48	↑ infected cell viability; ↓ HIV-1 integrase activity	↓ HIV 3'-end processing products	(*16*)
	H9 & MT-2 human T-lymphoblastoid	0.4	48	↓ HIV-1 integrase activity	↓ HIV integrase catalytic core	(*20*)
	MT-2 human T-lymphoblastoid	105	48	↓ HIV-1 reverse transcriptase activity	↓ HIV-1 envelope glycoprotein 120	(*29*)
	MT-2 human T-lymphoblastoid	0.4	72	↑ infected cell viability; ↓ HIV-1 reverse transcriptase activity; ↓ HIV-1 reverse transcriptase release	N/A	(*23*)
	MT-2 human T-lymphoblastoid	25	72	↓ HIV-1 integrase replication	N/A	(*19*)
	MAGI human cervical epithelial carcinoma	100	52	↓ HIV-1 integrase activity;	↓ HIV-1 Tat protein synthesis	(*82*)
	H9 & MT-2 human T-lymphoblastoid	0.1-10	24	↓ viral replication; ↓ HIV-1 integrase activity	↑ integrase divalent cations chelation	(*17*)
	MT-4 human T-	70	2	↓ viral replication cycle;	↓ HIV-1 envelope	(*83*)

	lymphoblastoid			↓ viral entry	glycoprotein 120	
	MT-2 human T-lymphoblastoid	4.2	72	↑ infected cell viability; ↓ HIV-1 integrase activity	↑ long terminal repeat circle formation	(*21*)
	H9 & MT-2 human T-lymphoblastoid	50	1-4	↓ HIV-1 integrase activity	↓ integrase binding site activity	(*22*)
	H9 & MT-2 human T-lymphoblastoid	25	1	↓ HIV-1 integrase activity	↓ integrase-mediated catalysis	(*18*)
Glucose metabolism	L6 rat myotubes & insulinoma-derived ISN-1 pancreatic β cells	200 & 20-100	1-2	↑ glucose uptake; ↑ insulin secretion	N/A	(*11*)
	INS-1 pancreatic β-cells; L6 myotubes; isolated hepatocytes (rat)	105-210	1-3	↑ glucose uptake; ↑ insulin secretion	N/A	(*50*)
	HepG2 human hepatoma	100	24	↓ insulin resistance; ↓ NO & ROS	↑ GLUT2 translocation; ↑ Akt; ↓ MAPK; ↓ NF-κB	(*51*)
	HepG2 human hepatoma	100	24	↑ glucose uptake	↑ IRS-1; ↑ Akt; ↑ AMPKα; ↑ SIRT1; ↑ GSK-3β; ↓ CREB	(*52*)
Inflammation responses	RAW 264.7 mouse macrophage	4.2	4	N/A	↓ NF-κB; ↓ TNF-α	(*36*)
	Macrophage	N/A	N/A	↓ cell inflammation	↓ TNF-α	(*35*)
	Jurkat E6.1 human	17	4	N/A	↑ NFκB	(*44*)

	leukemic T-cell lymphoblast					
	Mouse peripheral blood mononuclear cells	0.5-4.2	4	↑ immune homeostasis	↑ IL-2; ↑ IFN-γ; ↓ IL-4	(*41*)
	BV-2 mouse microglia	80	4	↓ cell inflammation	↓ iNOS; ↓ COX-2; ↓ PGE2; ↓ IL-1β; ↓ TNF-α	(*33*)
	Human umbilical vein endothelial cells	12.5-100	24	↓ endothelial dysfunction; ↓ cell apoptosis; ↑ cell viability; ↓ ROS	↑ SOD; ↑ eNOS; ↓ Bax/Bcl-2; ↓ cleaved caspase-3; ↓ MAPK; ↓ NF-κB	(*42*)
	HT-29 human colorectal adenocarcinoma	42	12	↓ cell inflammation	↓ NF-κB; ↓ COX-2; ↓ IL-1β; ↓ IL-18	(*43*)
Oxidative stress	Human plasma	1	5	↓ Cu (II)-catalyzed LDL oxidation	N/A	(*84*)
	RAW264.7 mouse macrophage	16-32	20	↓ oxidative stress	↓ PGE2; ↓ TNF-α; ↓ IL-1β; ↓ NF-κB; ↓ Akt	(*47*)
	RAW264.7 mouse macrophage	N/A	20	↓ oxidative stress & ↓ NO	↑ GSH; ↓ iNOS; ↓ NF-κB	(*48*)
	L6 rat myotubes	5-50	1	↓ ROS	↑ GPx; ↑ SOD; ↑ p-AMPKα; ↑ PGC-1α	(*73*)
	RGC-5 rat retinal ganglion cells	0.025	24	↑ cell viability & ↓ ROS	↓ cleaved PARP; ↓ cleaved caspase-3	(*67*)
	RAW264.7 mouse macrophage	340	19	↓ oxidative stress	↓ MyD88; ↓ iNOS; ↓ TNF-α	(*65*)
	HepG2 human hepatoma	100	24	↓ NO; ↓ ROS	↓COX-2; ↓iNOS; ↓ NF-κB	(*51*)
	Daudi & Namalwa B lymphocyte; JeKo-1	21-105	12-48	↓ B cell activating factor belonging to the TNF	↓ NF-κB; ↓ IκB	(*85*)

	mantle cell lymphoma, THP-1 monocytes & HepG2 hepatoma (human)			family (BAFF)		
	BV-2 mouse microglia	80	4	↓ oxidative stress	↓ NF-κB; ↓ MAPK; ↑ Nrf2	(*34*)
	SH-SY5Y human neuroblastoma	50	24	↓ oxidative stress; ↑ cell viability	↑ Nrf2; ↑ HO-1; ↑ NQO-1; ↑ CAT; ↑ GSH; ↓ TNF-α; ↓ IL-1β; ↓ malondialdehyde	(*75*)
Others	HL-7702 human hepatocytes & HepG2 human hepatoma	20-200	48	↑ infected cell viability; ↓ HBV activity	↓ HBV surface & envelope antigen	(*86*)
	Caco-2 & HCT-116 epithelial colorectal adenocarcinoma (human)	105-315	24-48	↓ cell proliferation; ↑ cell apoptosis; ↓ telomerase activity	↑ DNA fragmentation; ↑ cleaved caspase-9; ↑ cleaved PARP	(*80*)
	HeLa cervical carcinoma & MCF-7 breast carcinoma (human)	0.05	24	↓ doxorubicin-induced cell death	N/A	(*87*)
	Human skin fibroblasts	2	24	↓ dermal fibroblasts senescence	↓ MMP-3	(*81*)
	3T3-L1 mouse preadipocytes	100	48	↑ cell apoptosis & ↓ mitochondrial membrane potential	↑ cleaved caspase-3; ↓ Akt; ↑ MAPK; ↓ JNK & ERK1/2	(*64*)
	3T3-L1 mouse preadipocytes	100	24	↑ free radical scavenging	N/A	(*76*)

↑ - increase; ↓ - decrease; N/A, not available.

Acronyms: Akt, protein kinase B; AMPKα, AMP-activated Protein Kinase α; Bax/Bcl-2, B-cell lymphoma 2-associated X/B-cell lymphoma 2; BSO, 1-buthionine-(S,R)-sulfoximine; CREB, cAMP response element binding binding protein; CAT, chloramphenicol acetyl transferase; COX-2, cyclooxygenase-2; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal–regulated kinase 1/2; GLUT2, glucose transporter 2; GPx, glutathion peroxidase; GSH, glutathione; GSK-3β, glycogen synthase kinase 3β; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus 1; 4-HNE protein adducts, 4-hydroxynonenal-protein adducts; HO-1, heme oxygenase; IFN-γ, interferon γ; IκB, inhibitor of kappa B; IL-1β, interleukin 1 beta; iNOS, nitric oxide synthase; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; MAGI, HeLa CD4+ HIV-1 LTR-β-gal cells; MAPK, mitogen-activated protein kinase; MMP-3, matrix metalloproteinase-3; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor κB; NQO-1, NAD(P)H dehydrogenase; Nrf2, nuclear factor erythroid 2–related factor 2; LDL, low-density lipoprotein; PARP, poly (ADP-ribose) polymerase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator α; PGE2, prostaglandin E2; ROS, reactive oxygen species; SIRT1, sirtuin 1; SOD, superoxide dismutase; Tat, trans-activator of transcription; TNF-α, tumor necrosis factor α.

Table 2.2 In vivo effects of chicoric acid in the treatment of various disorders.

Diseases	Species (sex)	Models	Dose (mg/kg/d)	Duration (days)	Effects	Suggested Mechanisms	References
Brain function	Swiss albino mice (M)	Restraint stress (Porsolt's swim stress & conical polypropylene tubes)	1-2 *p.o.*	14	↓ behavioral despair; ↑ learning ability; ↑ neurotransmitters	↑ norepinephrine; ↑ dopamine; ↑ 5-hydroxy tryptamine	(*66*)
	Sprague−Dawley rats & ICR mice (M)	Retinal damage (N-methyl-D-aspartate / optic nerve crush)	2 μg/eye intravitreal injection	7	↑ retinal ganglion cell viability	↓ cleaved PARP; ↓ cleaved caspase-3	(*67*)
	C57BL/6J mice (M)	LPS challenges	0.5 g/L drinking water	45	↓ memory impairment; ↓ amyloidogenesis	↓ amyloid β ($A\beta_{1-42}$); ↓ BACE1; ↓ MAPK; ↓ NF-κB	(*33*)
	C57BL/6J mice (M)	LPS challenges	0.5 g/L drinking water	45	↓ oxidative stress induced neuron damage	↓ MAPK; ↓ NF-κB; ↓ iNOS; ↓ IL-1β; ↓ TNF-α; ↑ Nrf2; ↑ HO-1; ↑ NQO-1	(*34*)
	C57BL/6J mice (M)	D-glactose challenges	100 *i.p.*	56	↓ neuron damage; ↓ hippocampus shrinkage	↓ amyloid β ($A\beta_{1-42}$); ↑ BDNF	(*75*)
Glucose metabolism	Wistar rats (M)	No challenge	3-30 *i.p.*	4	↑ insulin secretion; ↓ hyperglycemia	N/A	(*50*)
	Swiss mice (M)	Streptozotocin challenge	3 *i.p.*	2 hrs	↓ hyperglycemia	N/A	(*59*)

	Swiss mice (M)	Streptozotocin challenge	1-3 *i.p.*	2 hrs	↓ hyperglycemia	N/A	(*60*)
	C57BL/6J mice (M)	Streptozotocin challenge	60 drinking water	4	↓ hyperglycemia; ↓ pancreas apoptosis; ↑ insulin secretion	↓ JNK; ↓ Bax/Bcl-2; ↑ PDX-1	(*61*)
Lipid metabolism & liver function	C57BL/6J mice (M)	High-fat diet challenge	15-60 *p.o.*	8	↓ body weight; ↓ hepatic steatosis	↓ TNF-α; ↓ IL-6; ↓ COX-2; ↓ JNK; ↓ PPARγ; ↓ C/EBPα; ↓ FAS; ↓ ALT & AST	(*64*)
	C57BL/6J mice (F)	Hepatic steatosis (alcohol)	4 drinking water	4	↓ hepatic triacylglycerols	↓ iNOS; ↓ 4-HNE protein adducts; ↓ TNF-α; ↓ PAI-1; ↓ CD11c	(*65*)
	C57BL/6J mice (M)	Methionine- and choline-deficient diet challenge	10-30 *p.o.*	4	↓ hepatic lipid accumulation; ↓ lipid peroxidation; ↓ hepatic ballooning, steatosis & inflammation	↓ SREBP-1c; ↑ Nrf2; ↑ AMPK; ↓ TNF-α; ↓ MCP-1; ↓ FAS; ↓ ALT & AST	(*63*)
	C57BL/6J mice (M)	Streptozotocin challenge	60 drinking water	28	↓ hepatic injury	↑ glycogen; ↑ glycolysis genes (Gck, Pk & Pfk); ↑ AMPK	(*52*)
Inflammation	Sprague-Dawley	LPS challenge	20 *p.o.*	4	↑ phagocytic	↑ TNF-α; ↑ IFN-γ	(*45*)

	rats (M)				activity; ↑ NO		
	Swiss albino mice (M)	Restraint stress (conical polypropylene tubes)	2 *p.o.*	14	↑ Th1/Th2 homeostasis; ↑ lymphocyte proliferation & T cell population [cluster determinant 3 (+), 4(+) & 8(+)]	↑ CD28 & CD80; ↓ CTLA-4; ↓ CD152; ↓ IL-10; ↑ IFN-γ; ↑ IL-2; ↑ IL-12	(*88*)
	Sprague-Dawley rats (M)	Arthritis (collagen)	8-32 *p.o.*	28	↓ paw swelling; ↓ organ index of the thymus and spleen	↓ NF-κB; ↓ TNF-α; ↓ COX-2	(*40*)
	ICR mice (M)	Anaphylactic shock (compound 48/80)	20 *p.o.*	2 hrs	↓ mortality rate; ↓ histamine levels in blood serum	N/A	(*89*)

M – male; F - female; ↑ - increase; ↓ - decrease; N/A, not available; p.o., Per os (oral administration); i.p., intraperitoneal injection. Acronyms: Akt, protein kinase B; ALT, alanine aminotransferase; AMPKα, AMP-activated Protein Kinase α; AST, aspartate aminotransferase; BACE1, neuronal β-secretase 1; Bax/Bcl-2, B-cell lymphoma 2-associated X/B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; CD11c, integrin α X chain protein; CD28, cluster of differentiation 28; CD80, cluster of differentiation 80; CD152, cluster of differentiation 152; C/EBPα, CCAAT/enhancer binding protein α; COX-2, cyclooxygenase-2; FAS, fatty acid synthase; CTLA-4, cytotoxic T-lymphocyte associated antigen 4; 4-HNE protein adducts, 4-hydroxynonenal-protein adducts; HO-1, heme oxygenase; IFN-γ, interferon γ; IL-1β, interleukin 1β; iNOS, nitric oxide synthase; JNK, c-Jun N-terminal kinase; Keap1, kelch-like ECH-associated protein 1; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NF-κB, nuclear factor κB; Nrf2, nuclear factor erythroid 2–related factor 2; NQO-1, NAD(P)H dehydrogenase; PAI-1, plasminogen activator inhibitor-1; PARP, poly (ADP-ribose) polymerase; PDX-1, pancreatic duodenal

homeobox 1; PPAR γ, peroxisome proliferator activated receptor γ; ROS, reactive oxygen species; SREBP-1, sterol regulatory element-binding protein 1; TNF-α, tumor necrosis factor α; Th1/Th2, T helper cells 1/2.

2.3 Suggested molecular targets of chicoric acid

As discussed in previous sections, chicoric acid modulates genes, transcription factors, growth factors, enzymes, and proteins involved in important cellular processes, such as virus infection, chemoresistance, inflammation, and glucose metabolism (Figure 2.2) (*11, 42, 82, 85*). Among them, it is suggested that NF-κB and TNF-α are two major mediators of chicoric acid's activities.

NF-κB is a protein complex involved in cellular responses to stress, free radicals, heavy metals, and bacterial or viral infections through regulating DNA transcription, cytokine production, and cell survival (*37, 38, 90*). It is known that the activity of NF-κB is directly suppressed by binding with the inhibitor of κB (IκB) in the cytoplasm (*90*). In contrast, the phosphorylation of IκB releases and activates NF-κB (*90*). Chen et al. (*85*) reported that chicoric acid deactivates NF-κB by inhibiting the phosphorylation of IκB, indicating that IκB may be a major regulator for chicoric acid-mediated inactivation of NF-κB. Alternatively, NF-κB can be activated in combination with TNF-α (*37*). Several studies reported that chicoric acid decreased TNF-α production (*40, 47, 64, 65, 75*), which might be a contributing factor to the reduced activation of NF-κB. Chicoric acid-related deregulations of NF-κB and TNF-α were shown to alleviate conditions such as autoimmune disorders, restraint stress, hepatic steatosis and neuron damage known to be involved in cellular inflammation and associated diseases (*34-36, 40, 47, 63-65, 75*).

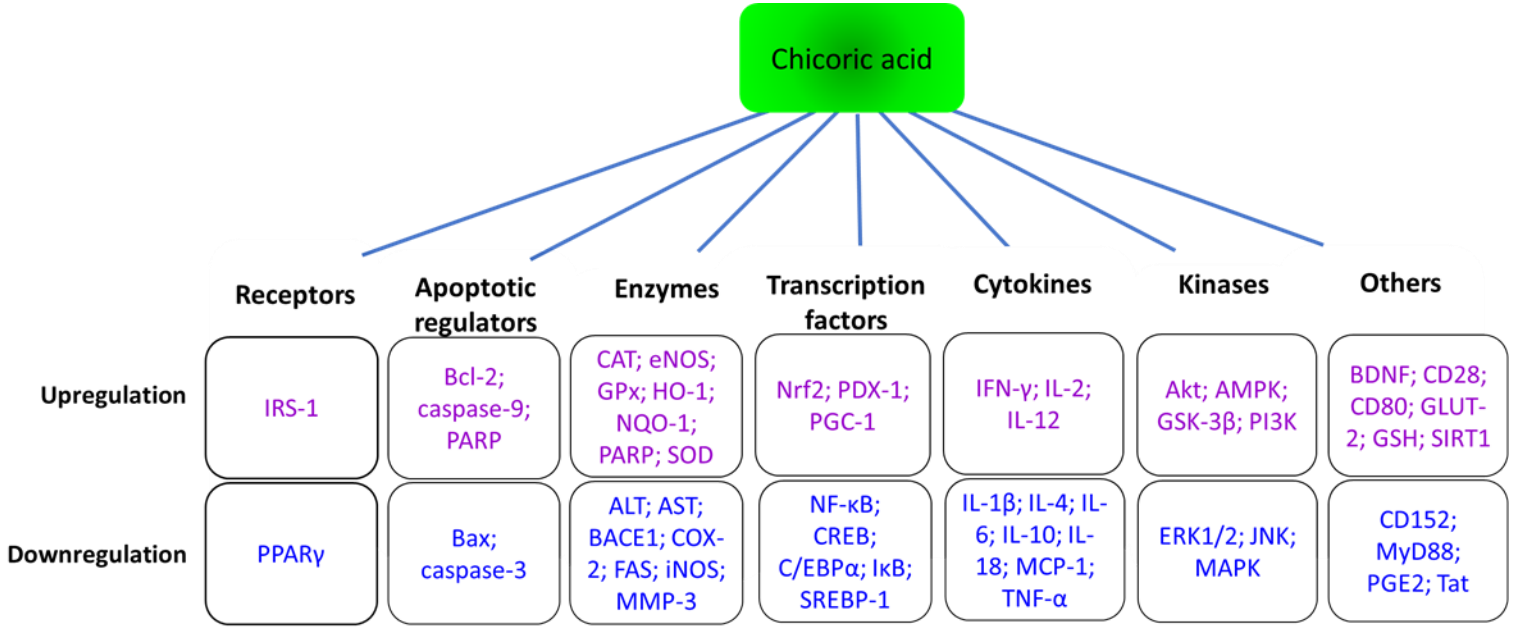


Figure 2.2 Molecular targets of chicoric acid. Akt, protein kinase B; ALT, alanine aminotransferase; AMPKα, AMP-activated Protein Kinase α; AST, aspartate aminotransferase; BACE1, neuronal β-secretase 1; Bax/Bcl-2, B-cell lymphoma 2-associated X/B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; CD28, cluster of differentiation 28; CD80, cluster of differentiation 80; CD152, cluster of differentiation 152; C/EBPα, CCAAT/enhancer binding protein α; COX-2, cyclooxygenase-2; ERK1/2, extracellular signal–regulated kinase 1/2; FAS, fatty acid synthase; HO-1, heme oxygenase; GLUT2, glucose transporter 2; IFN-γ, interferon γ; IL-1β, interleukin 1β; iNOS, nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; NF-κB, nuclear factor κB; Nrf2, nuclear factor erythroid 2–related factor 2; NQO-1, NAD(P)H dehydrogenase; PARP, poly (ADP-ribose) polymerase; PDX-1, pancreatic duodenal homeobox 1; PPAR γ, peroxisome proliferator activated receptor γ; SREBP-1, sterol regulatory element-binding protein 1; TNF-α, tumor necrosis factor α; Th1/Th2, T helper cells 1/2.

2.4 Pharmacokinetic study of chicoric acid

Chicoric acid is known to have relatively low absorption in rats (*91*). The oral administration of chicoric acid at 50 mg/kg body weight resulted in a peak plasma concentration of chicoric acid at 1.63 ± 0.25 mg/L after 4 h (*91*). Chicoric acid was mainly distributed in the liver, lung and kidney after oral administration for 3 h. Chicoric acid has a relatively long residence time and low body clearance, 18.58 ± 4.43 h and 2.80 ± 0.46 L/kg/h, respectively, in rats (*91*). All these findings help in better understanding the bioavailability and fate of chicoric acid.

2.5 Conclusion

The prevention of early-stage chronic diseases by food bioactive seems to be a promising strategy. Chicoric acid's multi-bioactivities suggest it has great potential in treating a number of metabolic disorders, including inflammatory responses, impairment of energy homeostasis, brain dysfunction, and immune disorders. Even though there are increasing number of studies reporting bioactivities of chicoric acid, these are still limitations to make a concrete conclusion due to differences on models, doses, and treatment durations used, as well as lack of pharmacokinetic study, including metabolism, of chicoric acid in humans. The research on chicoric acid is still at an early stage, so there are still many questions that need to be answered regarding its benefits to health. Thus, more studies are needed to guide the development of chicoric acid-based functional food products.

CHAPTER 3

OBJECTIVES OF THE PROJECT

The long-term goal is to develop chicoric acid as a functional food bioactive for the prevention and/or treatment for aging and type 2 diabetes. Previous studies reported that chicoric acid extends *C. elegans* lifespan and promotes glucose transport in hepatic cells (*7*). However, more experiments are needed to further investigate the effects of chicoric acid on aging-related phenotypes in *C. elegans* and the glucose uptake in other models. The object of this project is to determine the effect of chicoric acid on the aging-associated phenotypes of *C. elegans* and glucose uptake in muscle cells. The central hypothesis is that chicoric acid can protect *C. elegans* during aging as well as promote glucose uptake in muscle cells. The rationale of the proposed research is that by investigating the effect of chicoric acid on aging and glucose uptake as well as the underlying mechanisms, we will be able to better understand chicoric acid as a beneficial bioactive for the promotion of health span and prevention from type 2 diabetes.

The project aims are as follows:

Specific aim 1: Determine the effect of chicoric acid on aging and aging-related phenotypes of *C. elegans*. The hypothesis is that chicoric acid extends the lifespan of *C. elegans* and delays the decrease of aging-related phenotypes.

Specific aim 2: Investigate the effect of chicoric acid on glucose uptake in muscle cells and mice. The hypothesis to be tested is that chicoric acid promotes the glucose uptake in C2C12 myotubes and reduces the blood glucose level in mice.

CHAPTER 4

EFFECTS OF CHICORIC ACID ON AGING AND GLUCOSE UPTAKE

4.1 Effect of chicoric acid on aging in *C elegans* and the underlying mechanisms

4.1.1 Introduction

Aging is defined as a progressive decline of intrinsic physiological functions, resulting in an increased risk of death (*5*). Considering that aging is one of the key risk factors associated with many chronic diseases, slowing aging is of great importance to prevent age-associated diseases and improve the health span of life (*6*). Currently, food-derived bioactive compounds are drawing significant attention because of their anti-aging effects and the modulation of aging-related pathways (*92-95*), which provide potentially valuable preventive tools for aging and aging-related disorders.

Chicoric acid, one of the hydroxycinnamic acids, is a dicaffeoyl ester naturally found in the roots of many plants, such as chicory, purple coneflower, and basil (*4*). Chicoric acid is reported to have a number of bioactivities, including antioxidation, anti-inflammation, antivirus and immune-stimulating properties through multiple molecular pathways (*18, 33, 41, 52*). A previous study reported that chicoric acid extended the lifespan in *Caenorhabditis elegans* (*7*), however, the effects of chicoric acid on aging-related phenotypes and the mechanisms underlying these effects remain to be determined.

One recent study reported that chicoric acid regulates glucose metabolism via AMP-activated protein kinase (AMPK) activation (*52*). AMPK is an energy sensor that controls energy homeostasis through the regulation of AMP/ATP metabolism in cells (*4*).

Moreover, AMPK is considered important in the regulation of longevity as the increased activation of AMPK extended the lifespan through dietary restriction and/or enhanced stress resistance in *C. elegans* and *Drosophila melanogaster* (*96, 97*). *aak-2,* a homolog of the α-catalytic subunits of mammalian AMPK, is evolutionarily conserved in both *C. elegans* and mammals (*98*). Since AMPK has been reported to be activated by chicoric acid (*52*), it is plausible to assume that chicoric acid might regulate the lifespan of *C. elegans* via *aak-2*. In addition to *aak-2*, *skn-1*, encoding a homolog of nuclear factor erythroid 2–related factor 2 (Nrf-2) in *C. elegans*, was also reported to extend lifespan by regulating the oxidative stress response pathway (*99*). Therefore, the current research focused on determining the influence of chicoric acid on aging-related phenotypes in *C. elegans* and the role of AAK-2 and SKN-1 in this process.

4.1.2 Materials and methods

4.1.2.1 Materials

Chicoric acid (≥ 98%) was purchased from PufeiDe Biotech Co., Ltd (Chengdu, Sichuan, China). Household bleach used for bleaching adult nematodes during synchronization was obtained from Clorox company (Oakland, CA, USA). Fluorodeoxyuridine (FUdR), ampicillin, and carbenicillin, paraquat, and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) were ordered from Sigma-Aldrich (St. Louis, MO, USA). Rabbit antibodies for phospho-AMPK and α-tubulin, as well as goat anti-rabbit IgG-horseradish peroxidase (HRP) were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit antibodies for phospho-AMPK and α-tubulin,

as well as goat anti-rabbit IgG-horseradish peroxidase (HRP), were obtained from Cell Signaling Technology (Danvers, MA, USA).

4.1.2.2 *C. elegans*: strains and maintenance

The strains in the study were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA): wild-type Bristol N2, *aak-2* (*ok524*) *X*, *daf-2* (*e1370*) *III, eat-2*(*ad1116*) *II*, *daf-16*(*mgDf50*) *I*, *skn-1*(*zu135*) *IV, age-1*(*hx546*), and *ldIs7* (*skn-1b/c::gfp; rol-6*). All strains were grown and incubated at 20 °C on nematode growth media (NGM) plates with *Escherichia coli* OP50, except *daf-2,* which was maintained at 15 °C. The solutions including nematode growth medium (NGM), M9 buffer, and S-complete required for *C. elegans* culture were prepared according to the worm book (*100*). A synchronized culture was obtained as previously described (*101*). The chicoric acid stock solutions with 12.5, 25, and 50 mM were prepared using dimethyl sulfoxide (DMSO) as solvent. Stock solutions were diluted 1000 times resulting in final concentrations of chicoric acid at 12.5, 25, and 50 μM. These doses were based on a previous report on the effects of chicoric acid on life span extension in *C. elegans* (*7*). For lifespan analysis, L4 stage nematodes were transferred in Transwell-96 well plate (Corning Inc., NY, USA; n=10-15 worms per well, 15-18 wells/ treatment) and treated with 0.1% DMSO (control) or chicoric acid during the lifespan study (*94, 102, 103*). FUdR (120 mM), to prevent eggs from hatching out, was also added (*104*). The medium was changed every four days. The survivals were determined by a gentle touch-prod with a sterilized picker under a microscope (Nikon Instruments Inc., Melville, NY, USA) (*94, 103*). Worm viability was recorded every other day until all

worms died. The day that the treatment began is considered as day 0. The lifespan test in N2 was repeated for three independent trials.

4.1.2.3 Growth rate, body size, and moving speed determination

The growth rate of *C. elegans* was determined by quantifying the population of worms at three different developmental stages after 48 h chicoric acid treatments. Results were shown in percent of proportion of *C. elegans* in each stage (*94*). Body size and moving speed of *C. elegans* were measured by the WormLab tracking system (Allied Vision Technologies, Stadtroda, Germany) and analyzed by WormLab Software (MBF Bioscience, Williston, VT, USA) as published (*101*). Worms were transferred to the low-peptone NGM plates seeded with OP50 and were undisturbed for 30 min for acclimation before tracking (*94*). A 1-min recording (8.12 frames per second) was captured. The size of the worms (70-90/treatment) was measured by the WormLab Software after 48 h of treatments. The moving speed of worms was measured every other day from day 2 to day 12.

4.1.2.4 Progeny production and pumping rate determination

After 48 h chicoric acid treatments, two L4 staged nematodes/treatment were shifted to freshly prepared NGM plates with *E. coli* OP50 every day during the reproduction period. The number of progeny hatching out of eggs was counted and recorded daily (*94*). The pumping rate was obtained by quantifying pharyngeal contraction times of nematodes using a microscope (Olympus Corporation, Tokyo, Japan)

for 30 sec. The test was repeated three times with 12 randomly-selected nematodes per treatment (*94*).

4.1.2.5 Measurement of intracellular ROS and oxidative stress resistance

The intracellular reactive oxygen species (ROS) level was detected using DCFDA, a fluorescent probe commonly applied for ROS detection (*105*). After a 72-h treatment with DMSO (control group) or chicoric acid, day 1 adult nematodes were washed with M9 buffer three times to remove bacteria. They were next transferred into a 96-well plate, ~60 worms/well, with or without chicoric acid treatments in 50 μL of M9 buffer, following the mixture of 50 μL DCFDA solution (200 μM) to achieve 100 μM of the final concentration. A well with 100 μM DCFDA solution with no worms was used as the background, and worms treated with DCFDA solution and 5 mM paraquat were used as the positive control. The fluorescent readings were taken after 30 min of incubation at an emission/excitation wavelength of 530 and 485 nm by an Infinite 200 PRO microplate reader (Tecan Trading AG, Switzerland).

For oxidative stress resistance determination, day 1 adult worms were transferred into the 96 trans-well plate, 12-15 worms per well, containing 5 mM paraquat, an oxidative stress inducer. The oxidative stress resistance of C. elegans was determined by counting the survival percentage of worms under the exposure of paraquat for 2, 4, and 6 days with or without chicoric acid treatment (i.e., 25 μM) (*94*).

4.1.2.6 Quantitative real-time PCR

RNA was extracted in each sample using Trizol under RNase-free conditions, as

previously described (*101*). cDNA was prepared by reverse transcription of mRNA using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc, Middletown, VA, USA). The gene expression was quantified by QuantStudio 3 real-time PCR system using the TaqMan detection assay (Applied Biosystems, Waltham, MA, USA). *aak-2* (Ce02404259_g1), *nhr-49* (Ce02412666_g1), *skn-1* (Ce02407444_m1), *gsr-1* (Ce02444754_g1) and *pbs-5* (Ce02426103_g1), and the internal control *ama-1* (Ce02462726_m1) were ordered from Thermo Scientific (Middletown, VA, USA).

4.1.2.7 Intracellular localization of SKN-1::GFP

Transgenic strain *ldIs7* (*skn-1b/c::gfp; rol-6*) was used to detect the intracellular localization of GFP tagged SKN-1 protein. After two-day of chicoric acid treatment (25 μM), around 10-20 worms (L4 stage or young adults) were placed on each microscope slide coated with 2% agarose pad and anesthetized with 10 mM NaN_3 within 3 min (*94*). Worms treated with NaN3 (2%) for 15 min were used as the positive control. The cellular localizations of SKN-1 were detected by a Nikon Eclipse Ti-U (Nikon Instruments Inc., Melville, NY, USA). The nuclear translocation patterns of SKN-1::GFP were identified as 'low', 'medium', and 'high'. 'Low' refers to worms with almost no visible nuclear translocation; 'medium' refers to *C. elegans* with nuclear translocation in the partial body; 'high' refers to worms with nuclear translocation throughout the whole body, as shown in Figure 4.6D. Worms were counted and analyzed as percentages of nuclear translocation including both 'medium' and 'high' translocation (*103*).

4.1.2.8 Western blotting

The *C. elegans* protein extraction was obtained according to the protocol published with minor modification (*101, 106*). Worms were collected and washed four times with M9 buffer to remove bacteria. Then worms (approximately 6000 young adults per sample) were suspended by 500 μL PBS mixed with protease and phosphatase inhibitors (Beyotime Biotechnology, Shanghai, China). Samples were homogenized and centrifuged (12,000 × g) at 4 °C for 10 min to harvest the supernatants. After protein normalization, the samples with 5 × sample buffer were electrophoresed on a 10% SDS polyacrylamide gel and transferred onto a polyvinylidine fluoride membrane (*107*). After blocking with 5% skim milk, the membrane was incubated with rabbit monoclonal anti-phospho-AMPK and rabbit anti-α-tubulin antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Protein blots were pictured by a Tanon 5500 image station (Shanghai, China) with ECL Substrate Kit (Bio-Rad Co., Hercules, CA, USA). Images were quantified with ImageJ software.

4.1.2.9 Statistical analysis

Survival curves were analyzed by Log-rank (Mantel-Cox) tests by Graph Pad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). The data of nuclear translocation of SKN-1::GFP in Fig. 4.6D was analyzed by chi square tests by SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). For other data, differences between each treatment were analyzed using one-way ANOVA (Tukey's multiple-range test, SAS). Significance of differences was defined as P values of < 0.05.

4.1.3 Results

4.1.3.1 Chicoric acid extended the lifespan of *C. elegans*

Compared with the control, chicoric acid at 25 and 50 μM, but not 12.5 μM, significantly extended the lifespan of wild type nematodes (P<0.0001 for both; Fig. 1). There was no difference in lifespan between 25 and 50 μM chicoric acid treatments. Table 1 also exhibited the extended median lifespan of wild type N2 worms with 25 and 50 μM chicoric acid treatment, compared to the control. This was consistently observed from 3 independent experiments: (median lifespan as days) 18.7±0.3 for control, 23.3±0.9 for 25 μM chicoric acid, and 22.7±0.7 for 50 μM chicoric acid with P=0.0062 and P=0.0128 for 25 and 50 μM chicoric acid compared to the control, respectively. Thus, 25 and 50 μM were used to determine the aging-related phenotypes.

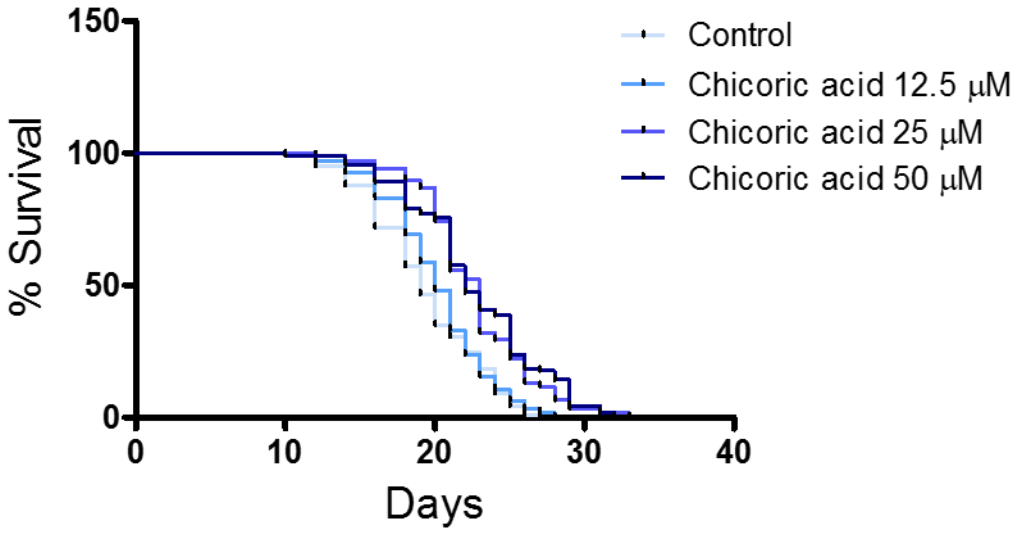


Figure 4.1 Survival of wild-type *C. elegans* treated with chicoric acid. *C. elegans* (N2) were treated with final concentrations of 0 (control), 12.5, 25, or 50 μM chicoric acid starting from L4 stage (day 0). The survivals were recorded every other day until all the worms died (n = 206–266/treatment). Log-rank (Mantel-Cox) test was used for statistical analysis. Significant differences of median/maximum lifespan compared between the control and the treatment groups were marked as ***P<0.001.

The physiological properties of *C. elegans* treated by chicoric acid after

synchronization were determined by growth rate, body size, and progeny (Fig. 4.2). After 48 h of treatment, 76–80% of the nematodes reached L4 stage or young adult stage, while 20–23% were at early/mid L4 stage (Fig. 4.2A). As shown in Fig. 4.2, no difference was observed in the growth rate, worm length, worm width, or progeny number of *C. elegans* treated with or without chicoric acid. The results suggest that the physiological properties of nematodes were not affected by chicoric acid.

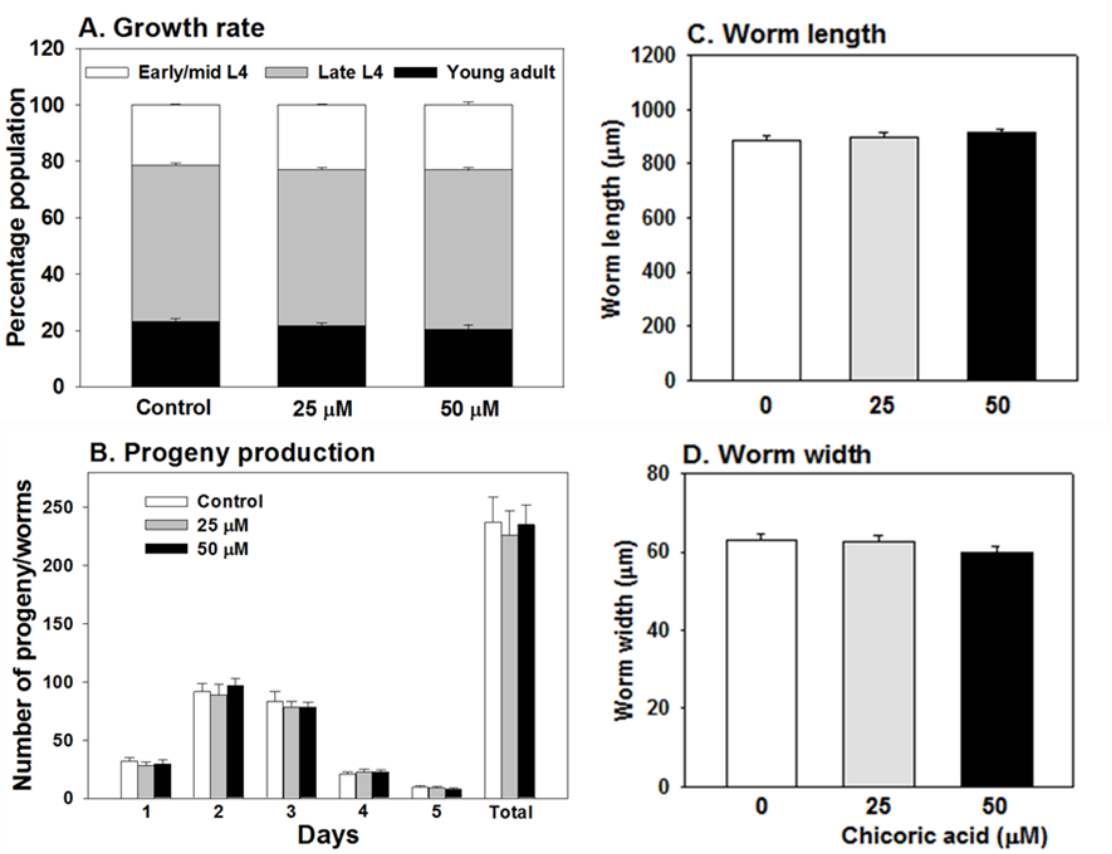


Figure 4.2 Effect of chicoric acid on growth rate, worm size and progeny production of *C. elegans*. (A) Synchronized L1 worms were raised on NGM plates with 0, 25 and 50 μM chicoric acid. After 48 h treatments, the number of worms at three developmental stages was counted and calculated in percent of proportion. (B) Synchronized L1 larval worms were treated with chicoric acid and the number of progeny hatching out from day 1 to day 5 were recorded. (C) Worm length and (D) width were measured by WormLab software after 48 h treatments from the L1 stage. Values are means ± S.E. (n = 3 plates and ~150 worms/plate for growth rate; n = 3 plates and 2 worms/plate for progeny production, n = 140–180 for worm size).

4.1.3.2 Effect of chicoric acid on the age-associated decline of phenotypes and ROS levels

Two typical markers of aging-relative phenotypes in *C. elegans* are pharyngeal pumping rate and locomotive activity (*102*). Thus, we next detected the effects of chicoric acid on these two markers (Fig. 4.3). The pumping rate as expected declined progressively with aging: 60% decline of the pumping rate in the control group from day 4 to day 12 (Fig. 4.3). Chicoric acid treatments with both 25 and 50 μM statistically delayed the decrease of pumping rate compared to the control (Fig. 4.3A). A significant difference was first observed on day 6, and a greater difference was found on day 12 (29.1% and 32.9% improvement, respectively, compared to the control). Aging resulted in the decline of moving speed by 76.4% on day 12, compared with that of day 2 in the control (Fig. 4.3B). Consistent with changes in the pumping rate, the decline in locomotive activity of the worms was lessened by chicoric acid treatments; On day 12, chicoric acid-treated *C. elegans* moved 59% and 86% faster than the control group, respectively (Fig. 4.3B).

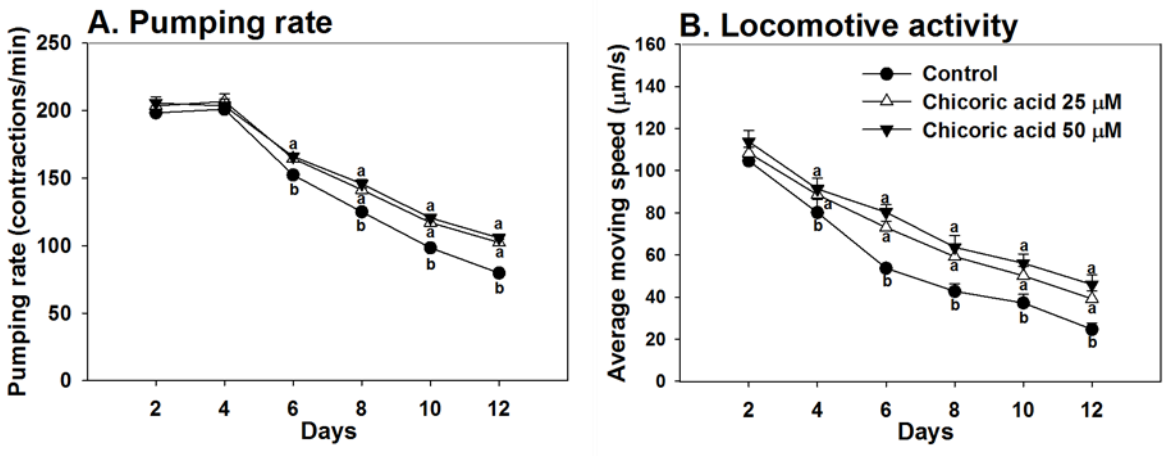


Figure 4.3 Chicoric acid ameliorated the age-related decline of movement and pumping rate. (A) Synchronized L1 *C. elegans* were treated with chicoric acid and pharyngeal

pumping was counted every other day until day 12. (B) Synchronized L1 *C. elegans* were treated with chicoric acid and moving speed was recorded by WormLab tracking system. Then the average moving speed of each worm was calculated using WormLab Software. Values are means ± S.E. n = 12 worms for pumping rate; n = 140–180 for worm speed. Values with different letters at each point in time are significantly different ($P < 0.05$).

Previous studies have reported that extended lifespan is closely associated with increased resistance towards oxidative stress (*93, 94, 96*). Since chicoric acid is known to possess anti-oxidative properties, the intracellular ROS levels of *C. elegans* were determined after the treatment of chicoric acid. The intracellular ROS levels in the chicoric acid treatment groups were significantly reduced (14.6% and 19.2% for 25 and 50 µM chicoric acid with P=0.0392 and P=0.0134, respectively) compared with the control (Fig. 4.4).

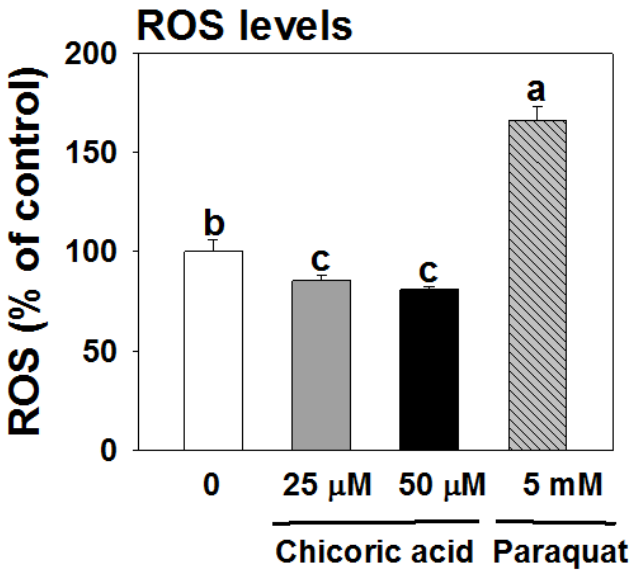


Figure 4.4 Effect of chicoric acid on the oxidative stress sensitivity of wild type N2 worms. Relative formation of reactive oxygen species (ROS) after 72 h of exposure to chicoric acid (0, 25 or 50 µM). Paraquat (5 mM) was used as the positive control. Values are means ± S.E. (n = 9; 60 worms/well). Values with different letters are significantly different (P<0.05).

4.1.3.3 Extended lifespan by chicoric acid required AAK-2 and SKN-1

Several studies reported that *aak-2* is an essential regulator in the aging of *C. elegans* through dietary restriction and/or stress resistance (*96, 108-110*). *aak-2* encodes a homolog of the catalytic-α subunit of AMPK in *C. elegans*. The deletion of *aak-2* shortens *C. elegans* lifespan, while the overexpression of *aak-2* results in *C. elegans* lifespan extension (*108, 109*). Since chicoric acid has been found to upregulate AMPK in hepatocytes (*52*), we determined the role of AMPK homolog in chicoric acid's effects on lifespan extension using *aak-2* loss-of function mutants. As shown in Table 4.1 and Fig 4.5A, the deletion of *aak-2* abolished chicoric acid-mediated lifespan extension, indicating the requirement of *aak-2* in *C. elegans*-lifespan extension by this compound.

In addition to *aak-2* , it is known that the insulin/IGF-1 signaling pathway is involved in aging in nematodes (*111, 112*). *daf-2* encodes for the insulin-like growth factor 1 (IGF-1) receptor, which is an important regulator for aging in nematodes (*113*). Mutants deficient in *daf-2* exhibit longer lifespan due to an extended dauer stage (*114*). One of the downstream targets of *daf-2* is *age-1*, another gene mainly regulating the longevity of *C. elegans* (*113*). Furthermore, DAF-2 and AGE-1 have been reported to inhibit DAF-16, an important transcription factor that regulates *C. elegans'* lifespan (*94*). To determine whether chicoric acid extends lifespan through the insulin signaling pathway, we tested *daf-2*, *age-1*, and *daf-16* null mutants. The *daf-2* deficient and *age-1* deficient mutants treated with 25 and 50 μM chicoric acid displayed significantly extended median lifespan compared to the respective controls ($P<0.0001$ for both; Table 4.1 and Fig 4.5B-C). Similarly, the median lifespan of *daf-16* mutants was significantly extended with the exposure of both 25 and 50 μM chicoric acid compared to the control;

14 days for the control (*daf-16* mutants) vs. 18 and 20 days with chicoric acid at 25 and 50 μM, with P=0.0012 and P=0.05, respectively (Fig 4.5D). These results indicate that chicoric acid may act independently of the insulin/IGF-1 signaling pathway in *C. elegans* (*113*).

In addition to AMPK and the insulin signaling pathway, several other molecular targets are known to regulate *C. elegans* lifespan, such as SKN-1 and EAT-2. SKN-1/Nrf-2 transcription factor has been found to mediate *C. elegans* lifespan extension mainly through the oxidative stress response pathway (*115, 116*). From Table 4.1 and Fig 4.5E, *skn-1* mutation abolished chicoric acid-induced lifespan extension, suggesting that *skn-1* is required for chicoric acid-mediated lifespan extension. Lastly, we tested *eat-2* mutant, which has defects of pharyngeal pumping that leads to prolonged lifespan via dietary restriction pathway (*117*). As shown in Table 1 and Fig. 4.5F, chicoric acid treatments shortened the median lifespan of *eat-2* deficient mutant, indicating the extended lifespan by chicoric acid might not occur through modulation of dietary intake. Since the observations that 25 and 50 μM chicoric acid led to similar effects on the aging and lifespan of mutants, we used 25 μM chicoric acid for the rest of the experiments.

Table 4.1 Effect of chicoric acid on median lifespan of wild type and mutant *C. elegans*.

Strain	Chicoric acid treatment (μM)	Median lifespan[a] (days)	Genetic requirement[b]
N2	Control	19	-
	25	23***	
	50	22***	
aak-2 (ok524) X	Control	12	Yes
	25	12	
	50	14	
daf-2 (e1370) III	Control	32	No
	25	36***	
	50	38***	
age-1 (hx546) II	Control	28	No
	25	32***	
	50	32*	
daf-16 (mgDf50) I	Control	14	No
	25	18**	
	50	20***	
skn-1(zu135) IV	Control	16	Yes
	25	18	
	50	18	
eat-2(ad1116) II	Control	32	No
	25	24***	
	50	26*	

All strains were raised at 20 °C, except daf-2 (e1370) III, which was raised at 15 °C. Worms were treated with chicoric acid (25 or 50 μM) from the L4 stage (day 0). Floxuridine (FUdR) was added with a final concentration of 120 μM. The survivals were recorded every other day until all the worms died. [a] The median lifespan was referred to the time when survival rate dropped to 50%. *, **, and *** mean significant difference compare with their respective control at $P<0.05$, $P<0.01$ and $P<0.001$, respectively (n = 180–266 worms/treatment). [b] Significant differences were analyzed by Log-rank (Mantel-Cox) tests. Genetic requirement was defined as "Yes" at $P<0.05$ or "No" at $P>0.05$.

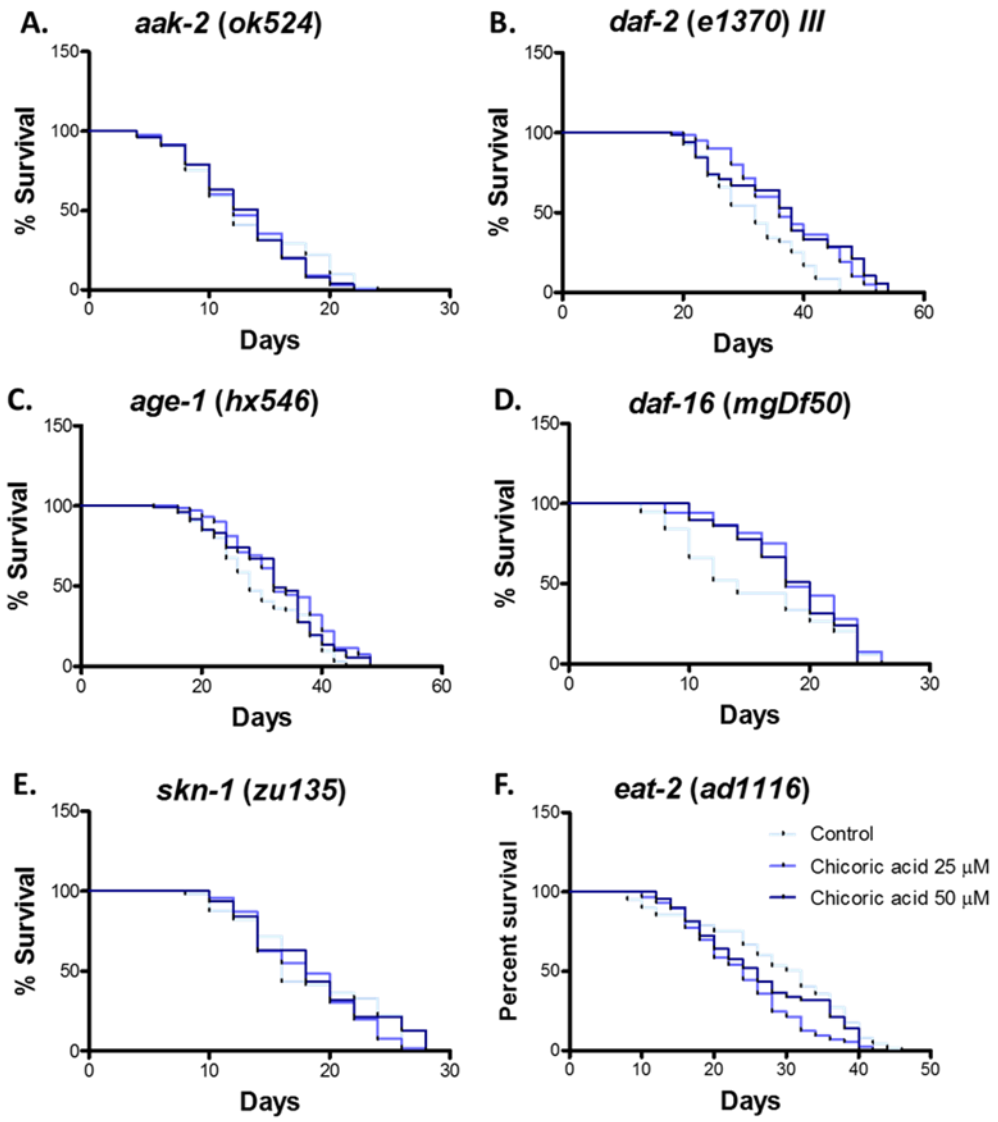


Figure 4.5. Survival of several mutant strains treated with chicoric acid. (A-F) Loss of function mutant strains in several genes related to lifespan regulation were treated with final concentrations of 0 (control), 25, or 50 μM chicoric acid starting from L4 stage (day 0). The survivals were recorded every other day until all the mutants died (n = 180–266 worms/treatment). Log-rank (Mantel-Cox) test was used for statistical analysis.

4.1.3.4 Chicoric acid might extend *C. elegans* lifespan via the regulation of AAK-2 and SKN-1.

Since the loss of aak-2 and skn-1 abolished chicoric acid-mediated lifespan extension, we determined the role of *aak-2* and *skn-1* in chicoric acid's effect on lifespan extension. First, we found that chicoric acid upregulated the mRNA expression of *aak-2* (Fig. 4.6A). Since it is known that AAK-2 can be regulated post-translationally (*97, 118, 119*), we measured the role of chicoric acid in phosphorylation of AAK-2 and found that chicoric acid significantly increased the phospho-AAK-2 level ($P<0.001$, Fig. 4.6B and C). We further determined the mRNA expression of a downstream target of AAK-2, *nhr-49*, a homolog of peroxisome proliferator-activated receptor alpha (PPARα) (*120*). As shown in Fig. 4.6A, chicoric acid treatment significantly increased expression of *nhr-49* compared to the control ($P=0.0066$).

Chicoric acid also significantly upregulated gene expression of *skn-1* (Fig. 4.6A). Similar to AAK-2, SKN-1 can be regulated post-translationally, which can be measured by nuclear translocation of SKN-1 in *C. elegans* (*121*). Chicoric acid treatment significantly promoted SKN-1 translocation from the cytoplasm to the nuclei ($P=0.0041$, Fig 4.6D), suggesting potential post-translational regulation of SKN-1 by chicoric acid. Next, we further tested two SKN-1 target genes, *gsr-1* (glutathione reductase) and *pbs-5* (proteasome beta subunit) (*122-124*), which were increased by chicoric acid treatment – a 1.7-fold and 2.4-fold increase over the control, with $P=0.0259$ and $P=0.0112$, respectively (Fig. 5A).

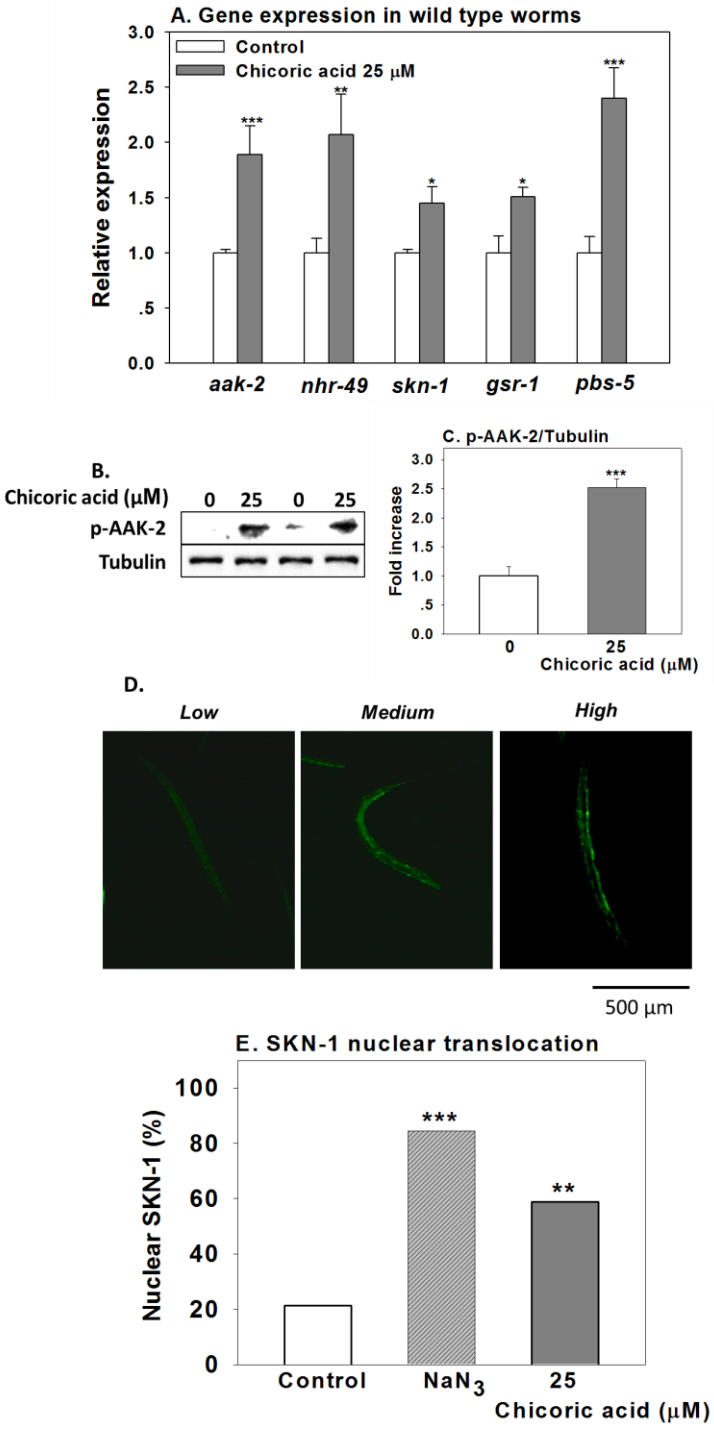


Figure 4.6 Chicoric acid might extend *C. elegans* lifespan via the regulation of AAK-2 and SKN-1. Synchronized L1 worms were treated with 0 or 25 μM chicoric acid for 48 h (A). The mRNA expression of *aak-2*, *nhr-49*, *skn-1*, *gsr-1*, and *pbs-5* were determined by real-time PCR. *ama-1* was used as an internal control. (B) Phospho-AAK-2 and tubulin were determined by immunoblotting. (C) p-AAK-2/Tubulin, phospho-AAK-2/Tubulin.

Values are means ± S.E. (n=3), *P<0.05, **P<0.01, and ***P<0.001. (D) Intracellular localization of SKN-1::GFP. The nuclear translocation patterns of SKN-1::GFP were identified as 'low', 'medium' and 'high'. (E) Nuclear translocation of SKN-1 in *ldIs7*; worms treated with chicoric acid were analyzed using the chi square test, and therefore do not show standard error bars (control, n=94; NaN3, n = 82; chicoric acid 25 μM, n = 107). A NaN3 (2%) treated group was used as the positive control. ** and *** mean significant difference compared with the control at P<0.01 and P<0.001, respectively.

4.1.3.5 Chicoric acid enhanced oxidative stress resistance in wild type N2 worms but not *aak-2* mutants

As *aak-2* has been reported to regulate oxidative stress resistance in nematodes (*119*), we then evaluated the influence of chicoric acid on oxidative stress responses of wild type worms and *aak-2* mutants. After 2, 4, and 6 days of paraquat exposure, the survival of chicoric acid-treated wild type worms was consistently improved over that of the control (12.2%, 12.8%, and 20.8% improvement for 25 μM chicoric acid with P<0.001, P=0.002, and P<0.001, respectively, Fig 4.7A). As shown in Fig 4.7B, the beneficial effect of chicoric acid against oxidative stress was abolished in *aak-2* mutants, suggesting an essential role of AAK-2 in chicoric acid-enhanced oxidative stress resistance in *C. elegans*.

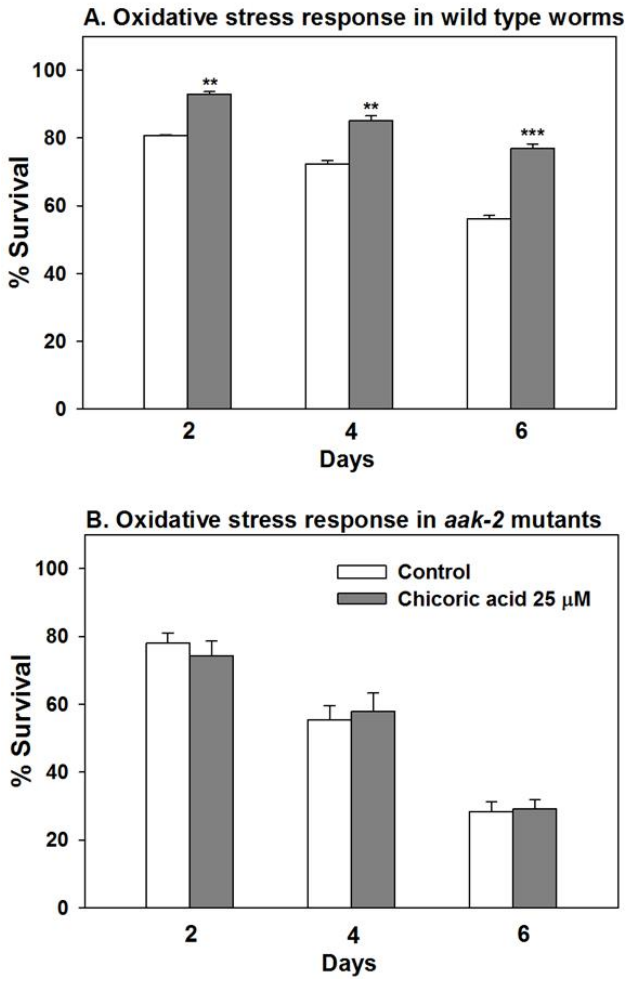


Figure 4.7 Chicoric acid enhanced the stress resistance of wild type worms but not aak-2 mutants. Wild type worms and aak-2 mutants (day 1 adults) were exposed to 5 mM paraquat with or without chicoric acid for 2, 4, and 6 days and survivals were counted. Values are means ± S.E. (n = 3; 120-145 worms/treatment), $^{**}P<0.01$ and $^{***}P<0.001$.

4.1.4 Discussion

In the current study, we found that chicoric acid at 25 and 50 μM significantly extended the lifespan of *C. elegans*. These findings are consistent with a previous study showing that chicoric acid at 25 μM or higher concentrations extended *C. elegans* lifespan (*7*). Here, we further investigated that chicoric acid-induced lifespan extension was accompanied by the prevention of age-associated declines of pumping rate and

locomotor activity as well as reduced oxidative stress sensitivity without influencing other physiological functions. The effects of chicoric acid was determined to be mediated in part by cellular energy sensor, AAK-2, and oxidative stress responsive transcription factor, SKN-1, but not via the insulin signaling pathway or dietary restriction.

C. elegans lifespan extension is closely associated with enhanced stress resistance, including oxidative stress (*125*). Increased oxidative stress, resulting from the excess production of free radicals, typically ROS, accelerates the aging of *C. elegans* (*70*). Here, we found that chicoric acid reduced ROS level in vivo and increased survivals under an oxidative stress condition, which is consistent with previous reports that chicoric acid reduced ROS and enhanced oxidative stress resistance in RGC-5 rat retinal ganglion cells and SH-SY5Y human neuroblastoma (*67, 75*). Antioxidative compounds, including chicoric acid, may directly act as ROS scavenger and/or indirectly activate stress-related signaling pathways, although based on the current results, it is not clear how chicoric acid elicits its effects on ROS.

The *aak-2* gene encodes an isomer of the AMPKα in *C. elegans* (*108*). A few studies have reported that *aak-2* is intimately involved in the control of *C. elegans* lifespan through the regulation of dietary restriction and/or stress resistance (*96, 108-110*). Based on the significant effect by chicoric acid on *eat-2* mutants, we determined that chicoric acid elicits its protective effects via stress resistance. In particular, Lee et al. (*119*) reported that *aak-2* mutations are hypersensitive to paraquat-induced oxidative stress, suggesting an important role of *aak-2* to *C. elegans* oxidative stress responses. This is supported by the current observation that *aak-2* deficiency completely abolished

the chicoric acid-mediated lifespan extension and survival during the oxidative stress conditions in *C. elegans* (Table 4.1 and Fig.4.5). The *nhr-49* is one of the downstream genes of *aak-2* (*120*), and it was suggested that NHR-49 may participate in oxidative stress responses and prevent ROS-induced toxicity in *C. elegans* (*121*). In fact, Moreno-Arriola et al. suggested that phospho-AAK-2 can regulate oxidative metabolism through increased transcriptional activity of *nhr-49* (*126*). Thus, chicoric acid may extend lifespan in part via a mechanism dependent upon *aak-2* and its target, *nhr-49*. However, since we were not able to determine the effects of chicoric acid on AAK-2 due to lack of available antibodies for AAK-2 of *C. elegans*, it is not conclusive that chicoric acid upregulated AAK-2 transcriptionally and/or post-translationally with the current results only.

skn-1 is also involved in chicoric acid-induced lifespan extension in *C. elegans*. SKN-1 starts regulating *C. elegans* lifespan as early as the postembryonic stage by activating cellular defense responses to oxidative stress (*127*). This was further supported by the fact that loss of function mutants in *skn-1* are sensitive to oxidative stress and the transgene of SKN-1::GFP rescues their embryonic developmental defect (*115*). It was reported that nuclear translocation of SKN-1 results in the higher cellular oxidative stress resistance in *C. elegans* (*121*). The current study showed that chicoric acid induced translocation of SKN-1 from the cytoplasm to nuclei with minimum enhancement of *skn-1* expression. This may suggest that chicoric acid's effect on post-translational regulation of SKN-1 contributes significantly to the antioxidation and lifespan benefits of chicoric acid in *C. elegans.* This was further confirmed by the observation of increased expression with chicoric acid treatment of two downstream genes of SKN-1, glutathione reductase (*gsr-1*) and proteasome subunit (*pbs-5*), which are essential for oxidative stress tolerance

in *C. elegans* (*122*). However, it is not clear if chicoric acid regulates SKN-1 directly or by regulating upstream regulators of SKN-1. In fact, AAK-2 was suggested to be the upstream of SKN-1 for metformin-mediated lifespan extension in a previous publication (*96*). Moreover, another study suggested that glycogen synthase kinase-3 (GSK-3) is the upstream of SKN-1 contributing to the increased resistance of oxidative stress in *C. elegans* (*128*). Thus, future experiments are needed to determine the target of chicoric acid on enhanced oxidative stress resistance.

Moreover, both AAK-2 and SKN-1 have been reported to regulate mitochondrial biogenesis against oxidative stress and contribute to longevity in *C. elegans*, suggesting the possible effect of chicoric acid on mitochondrial function (*98, 129*). One study reported that chicoric acid promotes mitochondrial biogenesis by increasing citrate synthase activity and the deacetylation of peroxisome proliferator-activated receptor-γ coactivator, which is directly activated by AMPKα (*7*). Thus, we cannot exclude the possibility that chicoric acid extends lifespan via a mitochondrial biogenesis-mediated mechanism.

Chicoric acid is metabolized to 2-caffeoyl-L-tartaric acid (cafraric acid) and caffeic acid by rat liver microsomes (*76*). A recent study reported that chicoric acid has low absorption in rats, with peak plasma concentration at 3.44±0.53 μM after 4 h of oral administration of chicoric acid at 50 mg/kg body weight (*91*). Thus, the 25 μM of chicoric acid used in the current study might be difficult to achieve in animals and humans. Moreover, currently the comparability of chicoric plasma concentration between

C. elegans and other *in vivo* models is not known; thus, it is important to determine the biological relevance of chicoric acid in humans.

In conclusion, chicoric acid significantly extended the lifespan of *C. elegans* in part through regulation of *aak-2* and *skn-1*. Because of the high conservation of these two genes between nematodes and mammals, these findings indicate that chicoric acid may have potential for promoting healthy aging as well as combatting age-related diseases in humans.

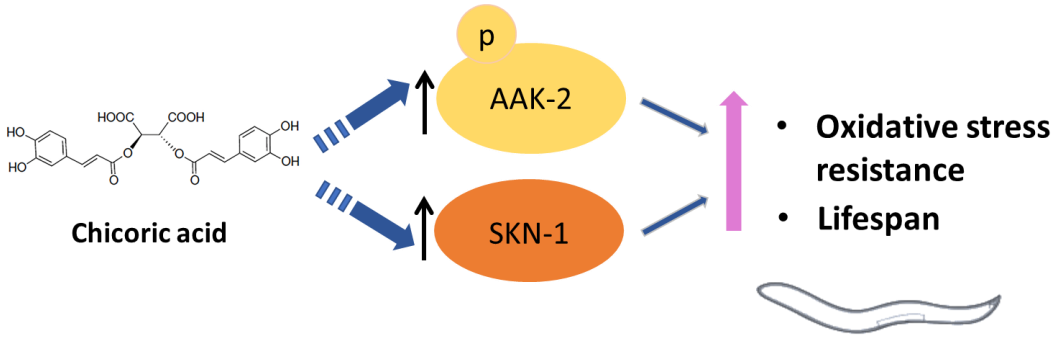


Fig. 4.8 The graphic contents. Chicoric acid significantly extended the lifespan of *C. elegans* and increase the oxidative stress resistance in part through regulation of *aak-2* and *skn-1*.

4.2 Effect of chicoric acid on glucose uptake and the underlying mechanism

4.2.1 Introduction

Type 2 diabetes mellitus (T2DM) has been recognized as a major public health challenge throughout the world in recent decades (*8, 9*). Impaired glucose uptake in muscle tissues is the primary associated with hyperglycemia in T2DM (*10*). Upon insulin signaling, protein kinase B, also known as Akt, is activated by phosphorylation that leads to translocation of glucose transporter 4 (GLUT4) to the plasma membrane, which subsequently promotes glucose uptake (*126, 130-132*). In addition to insulin signaling, several studies indicated that AMP-activated protein kinase α (AMPKα), which is known to regulate energy homeostasis (*133, 134*), also activates Akt, independent of insulin, and results in increased glucose uptake (*135, 136*).

Accumulating evidence suggests that many natural bioactive components have great potential to be used for the prevention or amelioration of T2DM (*126, 137*). Among natural bioactives, chicoric acid has been recently reported to have potential anti-diabetic effects (*11, 51, 73*). It is a naturally occurring dicaffeoyl ester often found in chicory plants and basil (Fig. 1A) (*138, 139*). One study reported that chicoric acid promoted glucose uptake in L6 muscle cells (*11*). More recently, another study revealed that chicoric acid regulated glucose homeostasis, stimulated the AMPKα pathway, and reversed insulin resistance in HepG2 cells (*52*). However, it is still not clear whether chicoric acid enhances glucose uptake in muscle cells via an AMPKα-mediated pathway. A better understanding of chicoric acid's impact in promoting glucose uptake in muscle cells would be significant given that muscle is responsible for approximately 75% of glucose disposal in the body (*140*). We used C2C12 myotubes, derived from murine

skeletal muscle cells and a C57BL/6J mice model to determine the role of chicoric acid on glucose homeostasis and its underlying mechanisms (*141, 142*).

4.2.2 Materials and methods

4.2.2.1 Materials

Chicoric acid (≥ 98%) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). C2C12 murine skeletal myoblasts were from American Type Cell Collection (Manassas, VA, USA). Bovine serum albumin, recombinant human insulin, and a penicillin/streptomycin mixture were purchased from Thermo Fisher (Waltham, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM), Kaighn's Modification of Ham's F-12 (F-12K), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, purity ≥ 93%), and horse serum (HS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dorsomorphin dihydrochloride (purity≥98%), also named Compound C, was purchased from MedChem Express (Monmouth Junction, NJ, USA). The protein content was determined using protein DC assay kits from Bio-Rad Co. (Hercules, CA, USA). Rabbit antibodies for phospho-Akt (Ser473), Akt (total), phospho-AMPKα, AMPKα (total), phospho-acetyl-CoA carboxylase (ACC) (Ser79), ACC (total), mouse antibody for GLUT4, goat anti-rabbit IgG-horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit antibodies for β-actin and Na^+/K^+-ATPase were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA).

4.2.2.2 C2C12 culture

C2C12 cells were cultured in a 5% CO_2 atmosphere at 37°C. Myoblasts were cultured and differentiated into multinucleated myotubes based on previous studies (*143*). After 6 days of differentiation, myotubes were treated with chicoric acid (12.5, 25, and 50 µM) for 24 h. For insulin treatment, 100 nM insulin was added into media for 30 min before the harvest of cells. A previous study reported that 20 µM Compound C inhibited the activity of AMPKα in primary hepatocytes (*144*). In the current model, 10 µM Compound C was able to inhibit AMPKα phosphorylation in C2C12 cells. Thus, 10 µM Compound C was used in this study. An MTT-based cell viability test was used to determine the cytotoxicity of chicoric acid (*145*). Briefly, C2C12 myoblasts were seeded in 96-well plates at a density of 1×10^6 cells/mL. Cells were treated with 12.5, 25, and 50 µM of chicoric acid for 24 h. The medium was then replaced with 5 mg/mL MTT in DMEM (without phenol red) for 4 h at 37°C. After incubation, cells were washed thrice with phosphate-buffered saline (PBS) and formazan crystals dissolved in DMSO were measured with microplate reader SpectraMax i3 (Sunnyvale, CA, USA) at 570 nm. Concentrations of chicoric acid tested (0-50 µM) were not toxic to C2C12 cells as shown in Fig. 4.9A.

4.2.2.3 Glucose uptake assay

For glucose uptake measurement, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), a fluorescent derivative of glucose, was used as described previously (*143*). Myoblasts were seeded in the 24-well plates. After 6 days of differentiation, myotubes were treated with various doses of chicoric acid in F-12K (5.5 mM glucose) serum free media for different time periods as shown the Figure legends.

C2C12 myotubes were then treated with and without 100 nM insulin for 30 min in a Krebs-Ringer buffer that was prepared as published previously (*146*). Insulin (30 min) stimulation was used to activate insulin signaling in C2C12 myotubes as previously described (*147*, *148*). After insulin stimulation, the 2-NBDG solution was added to each well at a final concentration of 200 µM, and the cells were incubated at 37 °C for 30 min. Cells were then rinsed thrice with ice cold PBS and the fluorescence was immediately measured using the SpectraMax i3 microplate reader at excitation/emission 465/540 nm. The cell lysate was prepared by adding 40 µl 0.1 M NaOH per well. The protein content of each well was determined by protein DC assay kits. The fluorescence was normalized by protein content.

4.2.2.4 Cell membrane protein extraction

Integral membrane proteins and membrane-related proteins from C2C12 myotubes were prepared using a Mem-PER Plus Membrane Protein Extraction Kit from Thermo Fisher Scientific (Waltham, MA, USA) according to the manufacturer's instructions. GLUT4 translocated to membrane and total GLUT4 were determined by immunoblotting.

4.2.2.5 Transfection of shRNA-AMPKα

Lentivirus-mediated short hairpin RNA (shRNA) targeting of the mouse AMPKα1 gene (shAMPKα) was established by Shanghai R&S Biotechnology Co. Ltd (Shanghai, China). Detailed information is provided in supplementary data (Supplementary Table S1 and Figure S2). The effective target sequence

ACGAGTTGACCGGACATAAAA in the mouse AMPKα1 gene (GeneID: NM_001013367.3) was selected, and a scrambled sequence: GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCA GTACATTT that had no significant homology to any mouse gene sequences was used as a negative control (shControl). Transfection of shRNA was performed based on the manufacturer's instructions. A virus counted with approximately 100 multiplicity of infection (MOI) was used to infect one well of cells in a 6-well plate for 12 h in a serum-free medium with polybrene (8 μg/mL), and the cell culture medium was then changed to a normal medium containing 10% FBS. After 48 h incubation, cells were treated with chicoric acid (0 or 25 μM) for 24 h. AMPKα protein levels were measured by Western blotting.

4.2.2.6 Western blotting

C2C12 cell lysate was prepared for immunoblotting as described previously (*107, 148*). Protein concentrations were determined by the protein DC assay kits. β-Actin was used as an internal control. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Protein blots were pictured by the Image Station 4000MM (Carestream Health, New Haven, CT, USA) with a Clarity Western ECL Substrate Kit (Bio-Rad Co.). Images were quantified with ImageJ software (version 1.49, National Institutes of Health, MD, USA).

4.2.2.7 AMP/ATP assay

The measurement of AMP and ATP from C2C12 myotubes was conducted using

a mouse AMP/ATP ELISA kit (Shanghai Fan Ke Biotechnology Co., Ltd., Shanghai, China). Briefly, C2C12 cells were treated with chicoric acid (25 μM) for 24 h, followed by washing with cold PBS twice at 4°C. Then cells were diluted by PBS to reach the final concentration 1 x 10^7 cells/ml. The diluted cells were lysed with a lysis buffer according to the manufacturer's instructions. The supernatant was obtained by centrifugation for 20 min at the speed of 3000 rpm. Then the supernatant of cell lysate was taken to detect the levels of AMP and ATP following the protocol of the ELISA kit. The absorbance of each sample was read by the Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450nm after adding a stop solution for 15 min.

4.2.2.8 Glucose tolerance test (GTT)

Male C57BL/6J mice (8 week) were obtained from the Laboratory Animal Research Center of Jiangsu University (Jiangsu, China) and maintained on a 12 h light/dark cycle with free access to food and water for 2 weeks. Institutional guidelines (serial No. UJS-LAER-2018042301) for animal care and use were followed, and the animal protocol was approved by the animal ethics committee of Jiangsu University. After adaptation, the mice were divided into two groups (6-8 mice/group). For the treatment groups, mice received a daily oral administration of 5 mg/kg BW chicoric acid for 5 days, based on a previous publication (*59*). For the control group, mice will be administrated with saline solution orally. Mice body weight will be measured before glucose tolerance test (GTT). On the fifth day, all mice will be fasted for 12 h following the first measurement of blood glucose from the tail vein by a blood glucose meter (0 min, ACCU-CHEK, Jiangsu, China). Then, all mice will be subjected to an intraperitoneal

GTT (2 g glucose/kg BW). The blood glucose levels will be further monitored at 15, 30, 60, and 120 min. The areas under the curves (AUCs) were calculated with Sigma Plot (version 11.0, Systat Software, Inc, San Jose, CA, USA).

4.2.2.9 Statistical analysis

SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was used for all data except for Fig. 4.9B, Fig. 4.10A, Fig. 4.11B, Fig. 4.13, and Fig. 4.14, where two-way ANOVA with least squares means statement was used. When there were no interactions between chicoric acid and another factor (insulin or time), no letter was used in the figures. If there were significant interactions between chicoric acid and another factor (Compound C or shAMPK), letters were used in the figures to present the differences between each group. The Tukey−Kramer method was applied for the multiple comparisons among the groups. A difference was considered significant at $P<0.05$.

4.2.3 Results

4.2.3.1 Chicoric acid promoted glucose uptake

When overall glucose uptake was measured by using 2-NBDG from C2C12 myotubes, there were significant effects of insulin ($P=0.0006$) and chicoric acid ($P<0.0001$) without any significant interaction between insulin and chicoric acid (Fig. 4.9A). Glucose uptake in insulin-stimulated groups was 20%-25% higher than in non-insulin-stimulated groups. This is consistent with previous reports that less than 30% increase of glucose uptake after insulin stimulation with 2-NBDG (*149*, *150*). The exposure of

C2C12 cells to chicoric acid at the concentrations of 25 and 50 µM resulted in significant increases in glucose uptake regardless of insulin stimulation compared to the controls (P= 0.001 and P=0.009, respectively, Fig. 4.9B). The effect of increased glucose uptake by chicoric acid (25 µM) peaked after a 24 h treatment, and was significantly 36% greater than the control group (P<0.0001; Fig. 4.9C); thus, a 24 h chicoric acid treatment was used for the subsequent experiments.

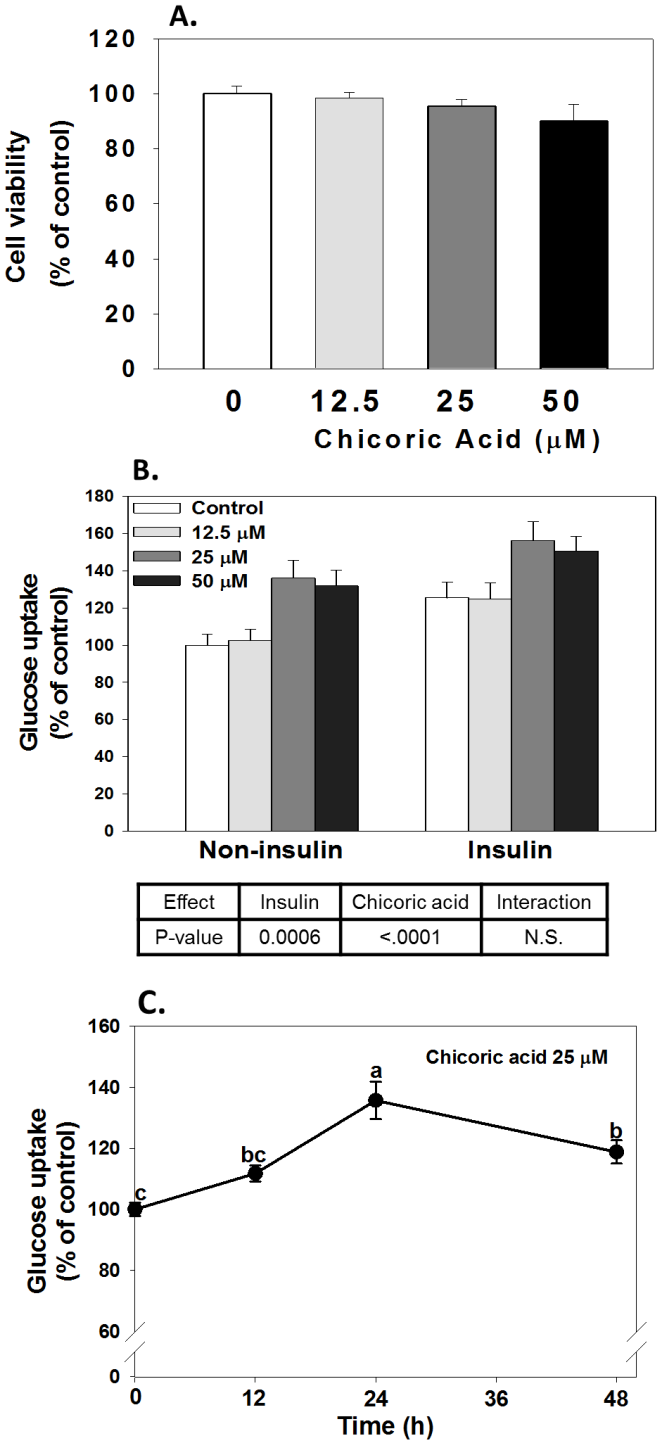
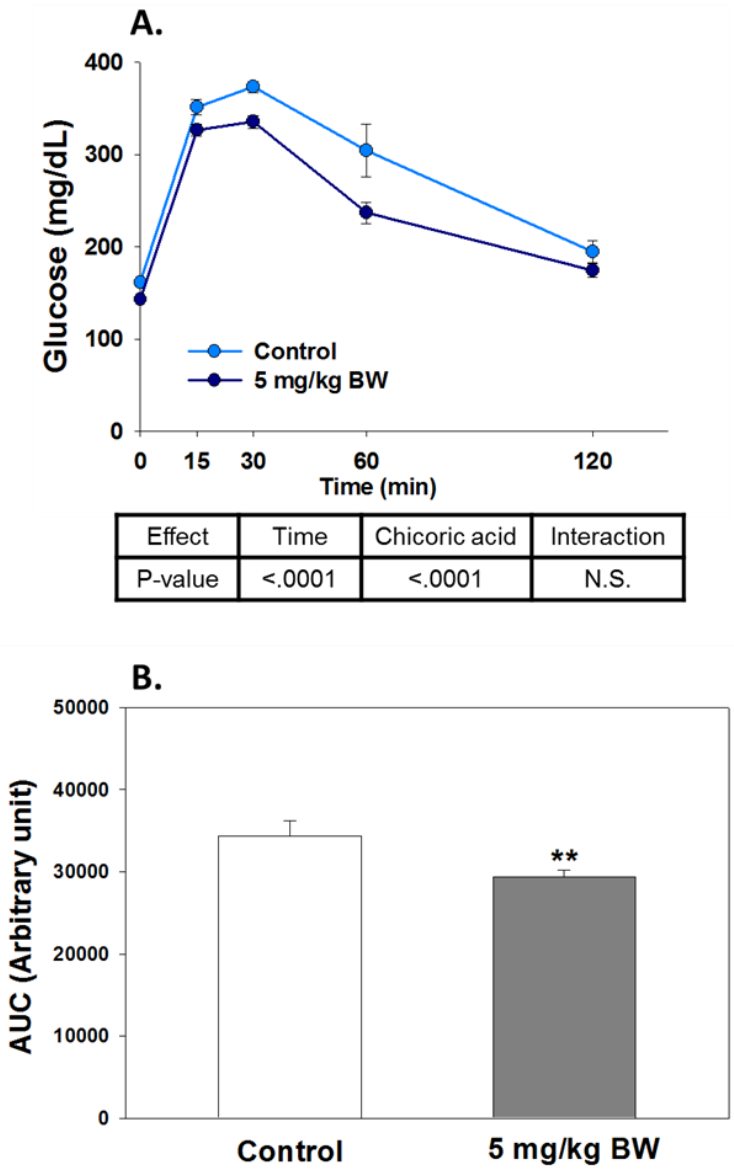


Figure 4.9 Chicoric acid promoted glucose uptake in C2C12 myotubes. (A) Concentrations of chicoric acid tested (0-50 μM) were not toxic to C2C12 cells. (B) Glucose uptake was measured from myotubes that were treated with chicoric acid (12.5, 25 or 50 μM) for 24 h, followed by a 30-min treatment with or without 100 nM of insulin. (C) Myotubes were treated with 25 μM chicoric acid for 12 h, 24 h or 48 h in F-12K (5.5

mM glucose) serum free media, respectively. Since glucose uptake was induced by chicoric acid independent of insulin, no insulin stimulation was conducted in this test. After washing thrice, 2-NBDG solution was added to each well at a final concentration of 200 µM, and the cells were incubated at 37 °C for 30 min. Cells were then rinsed thrice with ice cold PBS and the fluorescence was immediately measured using the SpectraMax i3 microplate reader at excitation/emission 465/540 nm. Numbers are mean ± S.E. (n=5). Means with different letters are significantly different at $P<0.05$.

We also conducted in vivo glucose tolerance tests (GTT) to determine if chicoric acid supplementation would have the similar results. There were significant effects in GTT for time ($P< 0.0001$) and chicoric acid ($P< 0.0001$) without any significant interaction between time and chicoric acid (Fig. 4.10A). Chicoric acid treatment (5 mg/kg BW for 5 days) led to a 14.5% reduction of the overall blood glucose level compared to the control ($P< 0.0001$; Fig. 2A). The areas under the curves (AUCs) in GTT further confirms that chicoric acid treatments improved glucose tolerance in mice ($P= 0.0065$, Fig. 4.10B).



Effect	Time	Chicoric acid	Interaction
P-value	<.0001	<.0001	N.S.

Fig. 4.10 Effects of chicoric acid on glucose tolerance test (GTT). Mice were administrated with (0 and 5 mg/kg BW/day) chicoric acid orally for 5 days. (A) Blood was collected from the tail vein, and glucose levels were measured at 0 min, then the glucose solution (2 g/kg BW) was administered by intraperitoneal injection and blood glucose level were determined at 15, 30, 60 and 120 min. (B) Area under the curve (AUC). Values are means ± S.E. (n = 6), ***P*<0.01. Means with different letters are significantly different at *P*< 0.05.

4.2.3.2 Effects of chicoric acid on Akt activation and GLUT4 translocation in C2C12 myotubes

It is known that the activation of Akt (phosphorylated-Akt, p-Akt) and GLUT4 translocation to the plasma membrane directly regulate glucose uptake in muscle cells (*9, 131*). Thus, we examined the effects of chicoric acid on the phosphorylation of Akt and GLUT4 translocation from C2C12 myotubes. Fig. 4.11A and 4.11B show that the ratio of p-Akt/Akt (as an indicator of activation of Akt) was significantly increased by insulin ($P<0.0001$) and chicoric acid ($P=0.0003$), without any interaction between insulin and chicoric acid. These results are consistent with the results in Fig. 4.9A that chicoric acid promoted glucose uptake in an insulin-independent manner. From these results, we selected 25 μM chicoric acid to determine its effect on GLUT4 translocation since this concentration had the highest activation of Akt, a 132% increase over the control in this model ($P<0.0001$; Fig. 4.11A). As shown in Fig 4.11C-E, the treatment of 25 μM chicoric acid resulted in a 28% increase in GLUT4 translocation to the cell membrane compared to the control ($P=0.0086$) without altering total GLUT4 expression. These results suggest that chicoric acid activated Akt independently of insulin and promoted GLUT4 translocation to the plasma membrane.

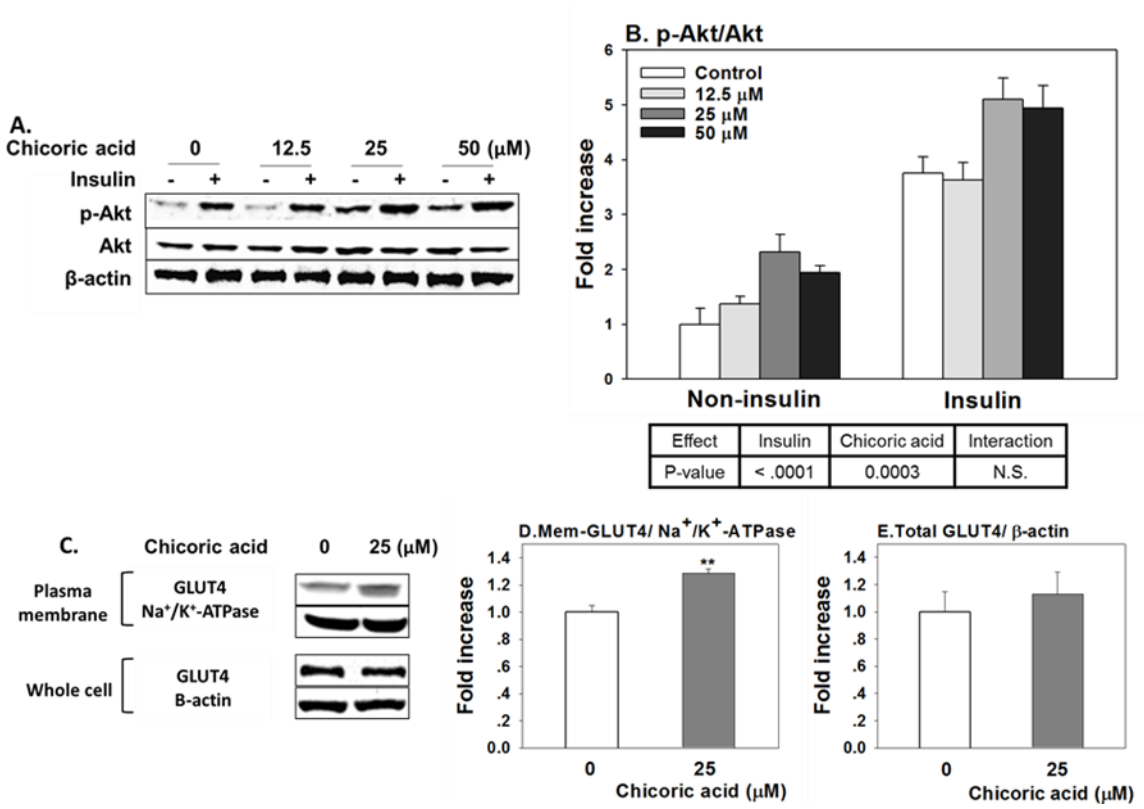


Figure 4.11 Activation of Akt and translocation of glucose transporter 4 (GLUT4) by chicoric acid in C2C12 myotubes. (A) Myotubes were treated with the indicated concentrations of chicoric acid for 24 h and/or 100 nM insulin for 30 min in F-12K serum-free medium. Then phosphorylation of Akt and total Akt was determined by immunoblotting. (B) p-Akt/Akt, phosphorylated protein kinase B/protein kinase B. (C) Chicoric acid induced translocation of GLUT4. C2C12 myotubes were treated with 25 μM of chicoric acid for 24 h. Then the protein of plasma membrane and whole cells were extracted to immunoblotting. (D) Mem-GLUT4/ Na^+/K^+-ATPase, GLUT4 on cell membrane/internal reference on cell membrane. (E) Total GLUT4/β-actin, GLUT4 in whole cells/internal reference in whole cells. Numbers are mean ± S.E. (n=3), $^{**}P<0.01$. Means with different letters are significantly different at $P< 0.05$.

4.2.3.3 Effects of chicoric acid on the AMPKα signaling pathway in C2C12 myotubes

Based on previous reports that chicoric acid promoted AMPKα activation in L6 myocytes (*73*), and AMPKα can activate Akt (*135*, *136*), we determine if the effect of chicoric acid (12.5 and 25 μM) on Akt was dependent upon AMPKα (Fig. 4.12). First,

we have confirmed that chicoric acid (25 μM) activated AMPKα (the ratio of p-AMPKα/AMPKα) with a 131% increase over the control (Fig. 4.12B). It was further confirmed that phosphorylation of ACC/ACC, one of main downstream targets of AMPKα, was consistently increased by chicoric acid treatment (Fig. 4.12C).

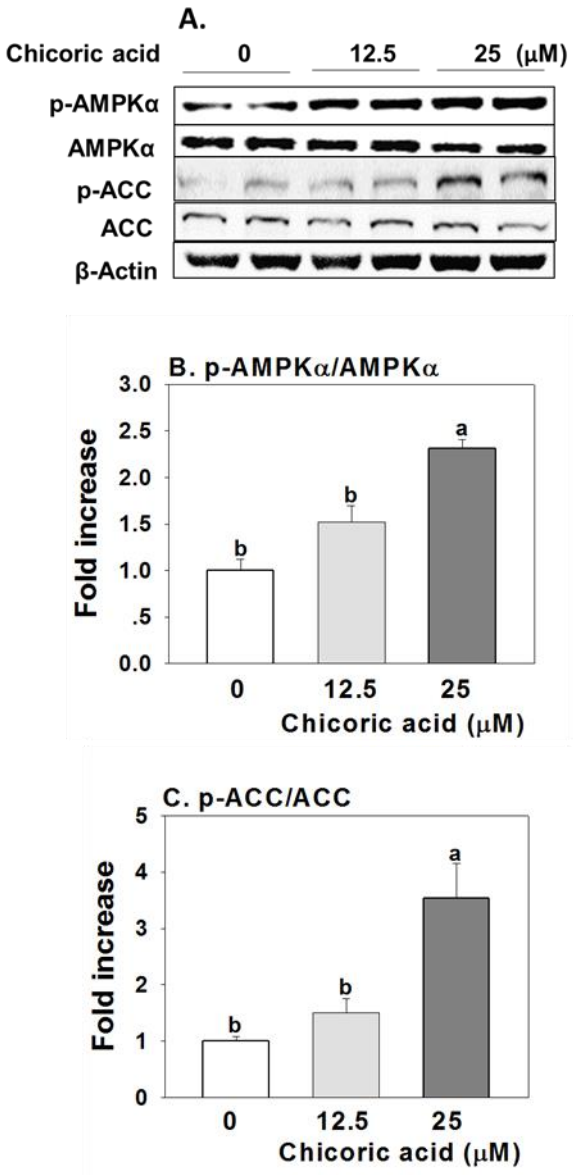


Figure 4.12 Activation of AMP-activated protein kinase α (AMPKα) pathway by chicoric acid in C2C12 myotubes. (A) Myotubes were treated with chicoric acid (12.5 μM or 25 μM) for 24 h in DMEM. Phosphorylation of AMPKα and ACC and the total were determined by immunoblotting. (B) p-AMPKα/AMPKα, phosphorylated AMP-activated protein kinase-α/AMP-activated protein kinase-α. (C) p-ACC/ACC,

phosphorylated acetyl-CoA carboxylase/acetyl-CoA carboxylase. Numbers are mean ± S.E. (n=4). Means with different letters are significantly different at $P<0.05$.

Next, we further proved that activation of AMPKα is involved in the effects of chicoric acid on activation of Akt by using two approaches: an AMPKα inhibitor (Compound C) and AMPKα knockdown cells. Compound C was regarded as a selective AMPKα inhibitor with no influence on kinases that have similar structures with AMPKα (*144*). Therefore, we used Compound C first to determine whether Akt activation by chicoric acid is mediated by an AMPKα-dependent pathway (*144*). Both Compound C and chicoric acid had significant effects on Akt phosphorylation, with a significant interaction between Compound C and chicoric acid ($P= 0.049$). The phosphorylation of AMPKα by chicoric acid was inhibited after treatment with Compound C (10 μM) as expected (Fig 4.13A-B). Chicoric acid consistently upregulated Akt phosphorylation without Compound C ($P= 0.003$), while chicoric acid induced Akt activation was abolished by Compound C (Fig. 4.13A & C).

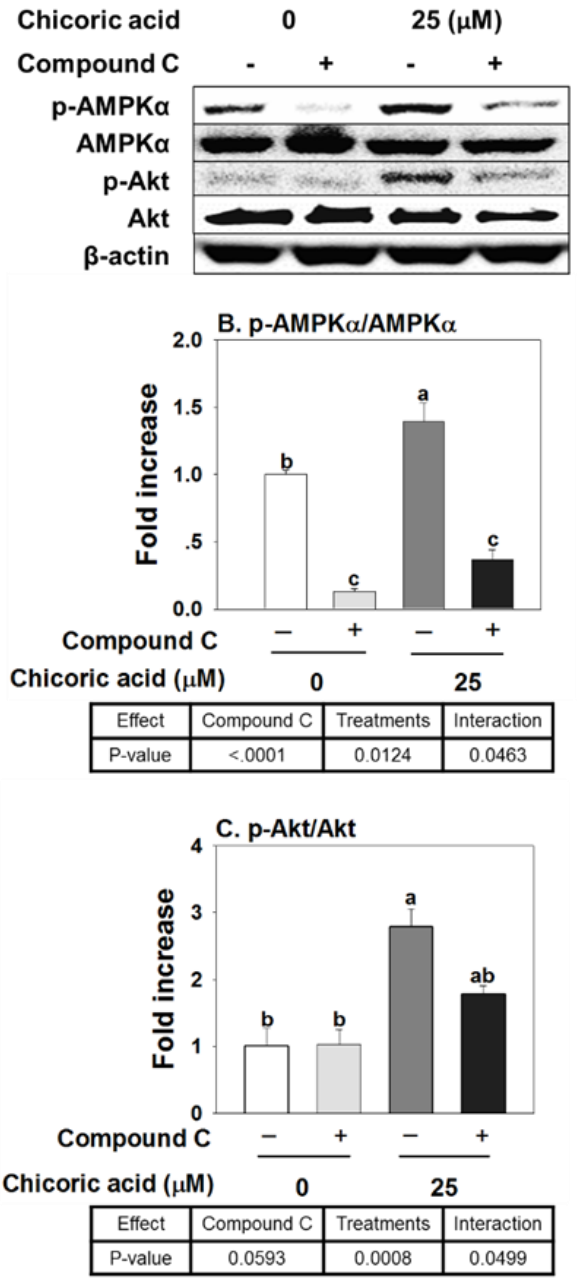


Figure 4.13 Increased glucose uptake and activation of Akt by chicoric acid were abolished by the inhibition of AMPKα. (A) Cells were treated with 10 μM compound C for 12 h, then myotubes were treated with 25 μM chicoric acid for 24h. (B) p-AMPKα/AMPKα, phosphorylated AMP-activated protein kinase-α/AMP-activated protein kinase-α. (C) p-Akt/Akt, phosphorylated protein kinase B/protein kinase B. Numbers are mean ± S.E. (n=3-5). Means with different letters are significantly different at $P<0.05$.

Next, we prepared AMPKα knockout cells to further confirm if chicoric acid had significant effects on Akt phosphorylation and glucose uptake. As shown in Fig. 4.14A and B, knocking-out AMPKα abolished the effects of chicoric acid on the activation of Akt. Consistently, increased glucose uptake by chicoric acid was abolished in AMPKα knock-out cells (Fig. 4.14C). These results indicate that chicoric acid increases the phosphorylation of Akt and glucose uptake through an AMPKα-dependent pathway.

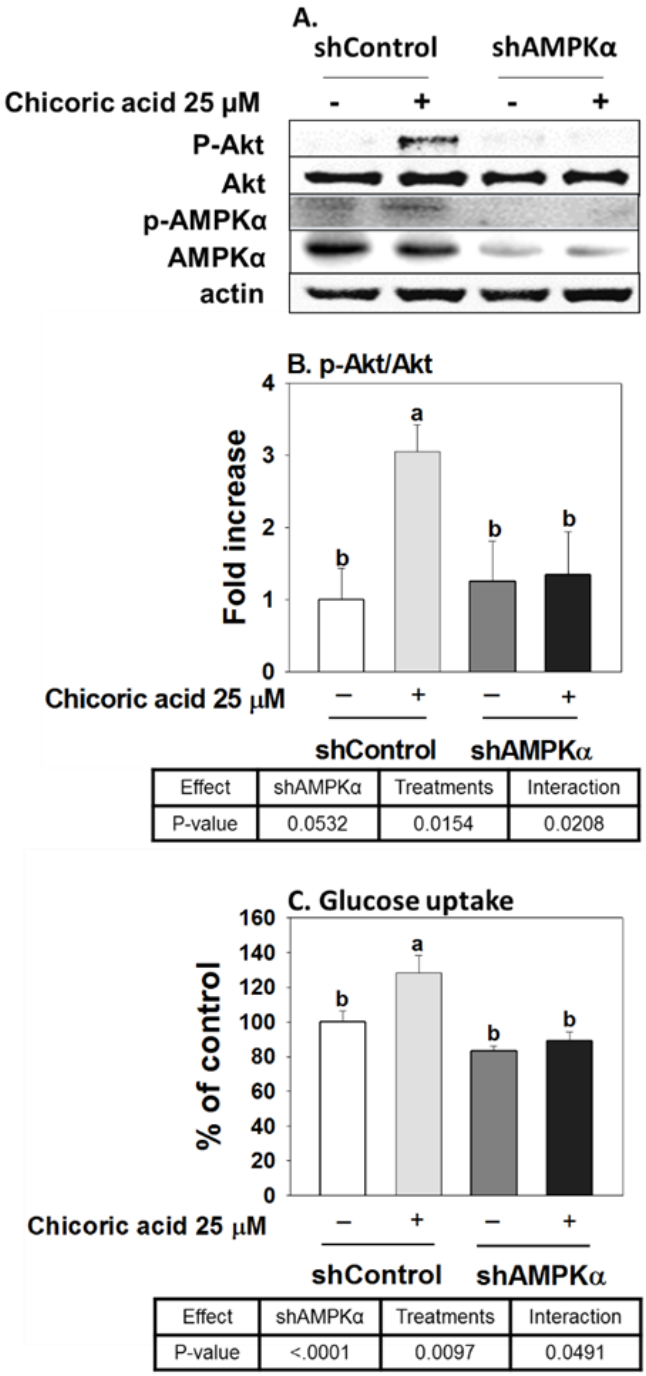


Figure 4.14 Increased glucose uptake and activation of Akt by chicoric acid were abolished by shAMPKα. (A) Cells were infected with lentivirus vectors expressing shRNA targeting mouse AMPKα1. Phosphorylation of Akt and the total Akt were determined by immunoblotting. (B) p-Akt/Akt, phosphorylated protein kinase B/ protein kinase B. (C) Glucose levels were determined in C2C12 myotubes treated with 0 or 25 μM chicoric acid for 24 h. Numbers are mean ± S.E. (n=3-5). Means with different letters are significantly different at $P<0.05$.

In aerobic respiration, an increase in AMP level has been reported to directly induce AMPKα activation (*151*). Thus, we further determined if AMPKα activation by chicoric acid is attributed to the enhanced AMP/ATP ratio. Figure 4.15 showed that the AMP/ATP ratio was increased by 133% in the 25 μM chicoric acid treatment group compared with the control (P<0.001), which suggests that chicoric acid activates AMPKα via regulation of the AMP/ATP ratio.

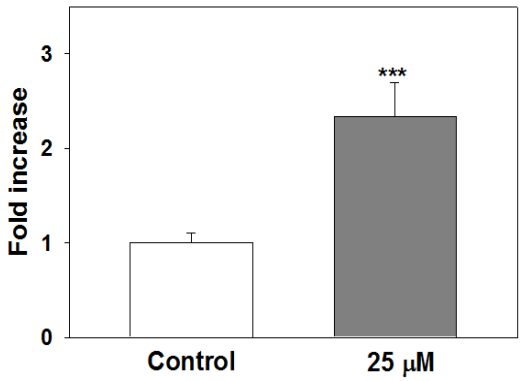


Figure 4.15 Chicoric acid enhanced AMP/ATP ratio in C2C12 myotubes. After 24 h of chicoric acid treatment, cells were lysed, and the supernatant lysate was taken to detect the levels of AMP and ATP using a commercial kit. The absorbance of each sample was determined by after incubation for 15 min. Values are means ± S.E. (n =3), ***P<0.001.

4.2.4 Discussion

Several phenolic secondary metabolites, such as chlorogenic acid and caffeic acid, have been used as antihyperglycemic compounds in pharmaceutical formulations (*11, 137*). Chicoric acid, a new phenolic ester, has been reported to have a beneficial effect on glucose transport in several studies (*51, 52, 59, 73*). Consistently, we demonstrated that chicoric acid increased glucose uptake and activated Akt, particularly at 25 μM, and these effects were dependent to AMPKα, but not insulin, in C2C12 myotubes. The

hypoglycemic effects of chicoric acid were further confirmed in vivo using mice at 5 mg chicoric acid/kg BW.

One in vivo study showed that chicoric acid (3 mg/kg BW) decreased the blood glucose level by 53% with a 120 min treatment in diabetic mice (*59*). Others reported that the extract from *Cichorium intybus* root, containing about 15 or 30 mg chicoric acid/kg BW, significantly decreased blood glucose level after 4 days in male Wistar rats (*152*). This is consistent with the current study which found that chicoric acid can significantly reduce glucose levels in male mice. Zhu et al. (*51*) reported that chicoric acid ameliorated glucosamine-induced insulin resistance through enhanced glucose uptake in HepG2 cells. Our current results indicate that chicoric acid increased glucose uptake, independent of insulin, in C2C12 myotubes (*51, 149, 153*). However, the current results are inconsistent with Tousch et al. (*11*), who found chicoric acid to increase glucose uptake only with insulin stimulation in L6 skeletal muscle cells . The inconsistency between the current results and that of Tousch et al. might be due to the different statistical analysis methods used for the latter (i.e. one-way, vs. two-way ANOVA in the current study). Alternatively, the inconsistency may derive from: using different cell lines (mice vs. rat muscle cell lines); different determination methods for glucose uptake; or a different length of treatment time (2-NBDG with 24 h treatment in the current study vs. [^{3}H] deoxyglucose with 1 h treatment in Tousch et al.).

AMPKα is a master regulator of energy homeostasis by activating proteins in catabolism pathways while turning off biosynthetic pathways (*154*). In cellular respiration, an increase in AMP level relative to ATP activates AMPKα, which further stimulates the acute upregulation of ATP production and the downregulation of non-

essential energy expenditure (*155*). Previous evidence also suggests that AMPKα is an important regulator of glucose transport, especially in muscle tissue by phosphorylation of insulin receptors, independent of insulin (*135*). Moreover, chicoric acid was previously reported to activate AMPKα, which was suggested to be involved in the regulation of glucose metabolism in HepG2 cells (*52*). In our study, a different cell line (C2C12 myotubes) was used, and we further found that chicoric acid increased the cellular AMP/ATP ratio, which might contribute to AMPKα activation. Prior study has suggested that increased cellular AMP level may attributed to an increase in mitochondrial respiration without a corresponding increase in ATP synthesis by oxidative phosphorylation, a process described as uncoupling of oxidative phosphorylation (*156*). It was reported that some caffeic acid phenethyl esters (CAPEs), the derivatives of caffeic acid, activated AMPKα through uncoupling of oxidative phosphorylation-induced increase in AMP level (*156, 157*). Since chicoric acid is structurally similar to CAPEs, we speculate that the increased AMP/ATP ratio by chicoric acid may from a mechanism similar to that of CAPEs. However, this still needs to be determined in future study.

Along with its hypoglycemic effect, there are reports of chicoric acid having other functional properties, such as antioxidation, anti-inflammation, and anti-fatty liver activities (*63, 84*). Chicoric acid has been considered a potent antioxidant agent due to its effect on inhibiting the accumulation of reactive oxygen species and the generation of inflammatory cytokines, such as nitrogen oxide, interleukin 6, and tumor necrosis factor-α (*51, 64*). Others have reported that chicoric acid promotes mitochondrial biogenesis by enhancing the citrate synthase activity and the deacetylation of peroxisome proliferator-activated receptor-γ coactivator, which is directly activated by AMPKα (*73, 154*). More

information about such properties will help us better understand chicoric acid as a functional food bioactive.

In conclusion, chicoric acid promoted insulin-independent glucose uptake and Akt phosphorylation by post-translational regulation of AMPKα in C2C12 myotubes, and improved glucose tolerance in the mice model. The current study demonstrated that chicoric acid is a potential antidiabetic nutraceutical that may be used for the prevention or treatment of diabetes.

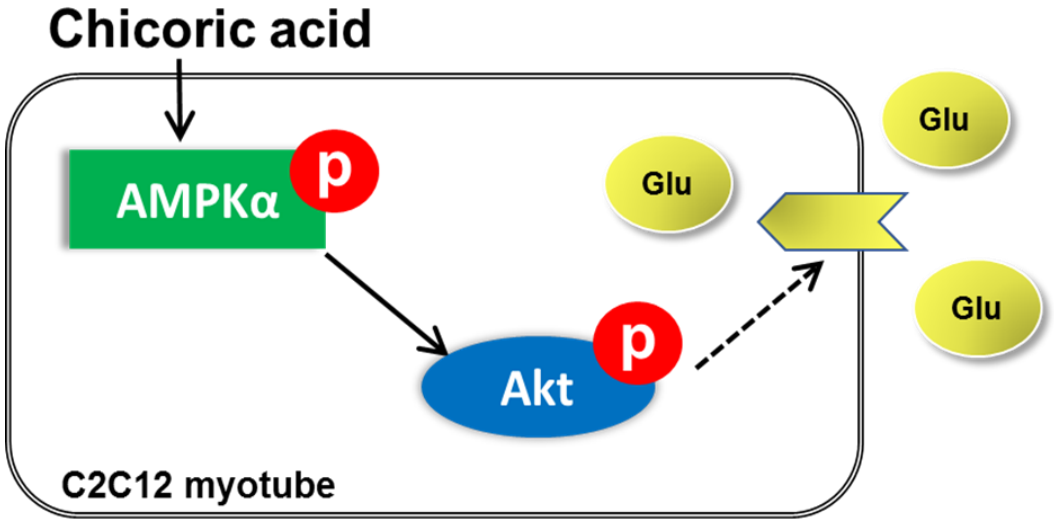


Fig. 4.16 The graphic contents. Chicoric acid promoted insulin-independent glucose uptake and Akt phosphorylation by post-translational regulation of AMPKα in C2C12 myotubes.

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