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The Link Between Coffee and Type 2 Diabetes: Chlorogenic Acid and Intestinal Glucose Absorption

by

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Abstract

Coffee ingestion is associated with reduced risk of type 2 diabetes (T2D). Coffee is the primary source of chlorogenic acid (CGA). The aim of this project was to examine the mechanisms by which CGA regulate blood glucose and insulin response, an impairment of which can lead to T2D. Sprague-Dawley (n=12) rats underwent a meal tolerance test with and without CGA in a cross-over design to measure blood glucose, insulin, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). CGA ingestion significantly attenuated blood glucose both and GIP response while no difference was noted with GLP-1 or insulin. Secondly, the NCI-H716 human colon cell line was incubated with CGA or its main metabolites (caffeic acid and ferulic acid); results showed no effect on GLP-1 release. This work shows that CGA can reduce postprandial blood glucose rise and has potential for development into an anti-diabetic agent.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AUC	Area under the curve
BSA	Bovine serum albumin
CA	Caffeic acid
CGA	Chlorogenic acid
CQA	Caffeoylquinic acid
FA	Ferulic acid
FBS	Fetal bovine serum
FFA	Free fatty acid
FPG	Fasting plasma glucose
FQA	Feroylquinic acid
GI	Glycemic index
GIP	Glucose-dependent insulinotrophic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
G-6-P	Glucose-6-Phosphate
HeBSS	HEPES buffered salt solution
HPLC	High performance liquid chromatography
KRB	Krebs-Ringer bicarbonate
MH	Meat hydrosylate
NEFA	Non-esterified free fatty acid
OGTT	Oral glucose tolerance test
PCG	Postchallenge glucose
PMSF	Phenylmethylsulfonyl fluoride
SGLT	Sodium glucose co-transporter
TG	Triglyceride

Chapter One: Introduction

1.1 Study Background

Type 2 diabetes (T2D) is a serious disease with the prevalence rapidly rising in much of the Western world. T2D is characterized by either the lack of sufficient insulin production or the inability to utilize insulin produced (1). Consequently, T2D results in an insufficient insulin response and elevated blood glucose; in the long term, this can lead to further health complications including cardiovascular disease, kidney disease, blindness and nerve damage (2-4). T2D is therefore associated with a lower quality of life as well as significant health care and economic burdens. By 2020, current statistics predict 3.7 million Canadians will have been diagnosed with T2D, costing our health care system $16.9 billion per year (5). While T2D is treatable, the disease progressively worsens and requires increased intervention (1, 6). Lifestyle changes such as weight management with exercise and diet are usually the first line of defence (7). These strategies are also used as preventative measures, as undergoing moderate intensity exercise for 30min/d provides a 30-50% reduced risk of developing T2D (8, 9). Specific dietary recommendations include reducing sugar and fat intake while increasing consumption of dietary fibre (10). Drug therapy is also commonly prescribed, yet anti-hyperglycaemic agents have side effects and some have serious contraindications such as increased risk of cardiovascular disease (11).

The risk of developing T2D associated with coffee consumption has been examined worldwide. In 2006 Greenberg and colleagues (12) identified 20 epidemiological studies examining this relationship, with 17 of those reporting a beneficial effect of coffee consumption on blood glucose levels. Since that article, an

additional 5 studies have found that coffee consumption is correlated with significantly reduced risk for developing T2D (11-15). Epidemiological studies also show coffee consumption to be correlated to reduced risk of T2D in a dose-dependent manner (13-17). A systematic review by Van dam & Hu (14) found relative risk reductions of 0.65±0.12 (95% confidence interval) with ≥6 cups per day. Risk reductions are seen with both decaffeinated and caffeinated coffee and since caffeine is known to impair insulin sensitivity, a component of coffee other than caffeine is likely responsible (18, 19).

Coffee beans contain thousands of constituents, including lipids, proteins, carbohydrates, vitamins, minerals and other components (16, 20-22). Given this, determining which compounds are responsible for the protective effects of coffee on T2D is difficult. Most coffee research has focused on caffeine as the bioactive ingredient and caffeine is known to inhibit muscle glucose uptake, thereby reducing insulin sensitivity and glucose tolerance (23). More recently however, findings that coffee and caffeine are not physiologically equivalent have increased the exploration of other coffee constituents (22, 24-27). Of particular interest is CGA, which are the main antioxidants in brewed coffee (24, 28, 29). CGA have been found to reduce intestinal glucose absorption and inhibit glucose-6-phosphatase *in vitro* (30, 31). Thus it is likely that these compounds are involved in mediating coffee's effects on blood glucose and insulin sensitivity.

1.2 Study Objectives

The purpose of this study was to determine the extent that CGA are involved in moderating the glycemic effects attributed to coffee. To accomplish this, 2 experimental models were explored: an *in vivo* acute feeding study using rats in a cross-over design

and an *in vitro* cell culture study using human colon cells incubated with/without CGA. The main outcome of the *in vivo* study was blood glucose, with several other biochemical markers affected by altered glucose assessed including plasma insulin, non-esterified free fatty acids (NEFA), glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP). For the *in vitro* study, the primary measurement was secretion of glucagon-like peptide-1, with glucose uptake also being determined.

The main hypothesis for this project was that CGA reduces the rate of glucose absorption along the small intestine. This was expected to result in a reduction of peak blood glucose rise after meal ingestion *in vivo* and/or reduced cellular glucose absorption *in vitro*. Consequently, levels of GLP-1 would be increased with CGA treatment compared to control both *in vitro* and *in vivo*, while GIP secretion would be lower in the animal experiment. Additionally, levels of insulin and NEFA were predicted to correlate with glucose levels in rats, with higher concentrations of insulin produced and lower levels of NEFA measured with increased blood glucose levels.

1.3 Thesis Composition

This thesis is composed of 5 chapters arranged as follows: Chapter 1 is an introduction to the thesis, describing the background for the research done; Chapter 2 consists of a review of the pertinent literature and presents the study objectives; Chapter 3 is a manuscript describing the acute *in vivo* effects of the compound of interest on glucose metabolism in a rat model; Chapter 4 is a manuscript describing cell culture experiments done to establish the *in vitro* effects of the compound on specific hormone release and Chapter 5 provides a general discussion and conclusion to the thesis. Chapters

3 and 4 each contain a short introduction, description of methods used, results and a discussion section specific to the study. References for the thesis in its entirety are found following Chapter 5.

Chapter Two: Literature Review

Coffee is a widely consumed beverage around the world, brewed and ingested in a variety of forms. It is estimated that ~50-60% of adults in North America consume coffee on a daily basis (32). American adults drank an average of 340mL/d coffee during 1999-2002, while coffee drinkers in Canada consumed an average of 650mL/d (32, 33). Recent epidemiological studies show coffee consumption to be correlated to large risk reductions in T2D. A summary of these studies can be found in **Table 1**. In small scale clinical trials, coffee consumption has also been linked to lower fasting glucose concentrations, an indicator of improved insulin sensitivity (34). Besides lowering disease incidence, coffee consumption may limit the progression of T2D. Examination of coffee consumption in individuals with impaired fasting glucose (6.1mmol/L $\leq$ fasting plasma glucose (FPG) <7.0mmol/L and postchallenge glucose (PCG) <7.8mmol/L) or impaired glucose tolerance (FPG < 6.1mmol/L and 7.8mmol/L $\leq$ PCG $\leq$ 11.1mmol/L) show a reduced risk of incident diabetes with reductions in odds ratios of 0.31 and 0.36 among past and current coffee consumers (35). In addition to T2D, habitual coffee consumption may delay the development of symptoms associated with the metabolic syndrome. Metabolic syndrome describes a combination of factors that increase risk for cardiovascular disease and diabetes, including high blood pressure, central obesity (waist circumference over a set value), elevated fasting plasma glucose, and altered lipid profiles (raised triglycerides and reduced HDL cholesterol levels). Hino and colleagues (36) found the frequency of the metabolic syndrome to decrease with increasing coffee consumption. Specifically, coffee consumption was correlated to lower waist

circumference, blood pressure, triglycerides and fasting plasma glucose levels. Of note, these are also risk factors towards the development of T2D. Given that coffee is already a popular, readily available and inexpensive beverage, its use in preventing and/or reducing T2D has widespread health implications. Understanding coffee's mechanisms of action as they relate to glucose management and insulin sensitivity will lead to increased knowledge of how this and other dietary factors alter T2D disease risk. Such information is useful in the development of dietary guidelines as well as the discovery of novel therapeutic targets and nutraceutical formulations. This thesis will focus on the physiological effects of a main coffee constituent, chlorogenic acid.

2.1 Chlorogenic acid

Chlorogenic acid are phenolic compounds responsible for the bitter taste of coffee (37). Phenols are abundant in the diet, found in all plant-based foods and beverages such as chocolate, nuts, fruits, coffee, wine and tea. A simple phenol refers to a compound with a single aromatic ring containing at least one hydroxyl group, while polyphenols have more than one of these rings (38). Phenols are commonly divided into classes that depend on their chemical structure, such as flavonoids and hydroxycinnamic acids (38, 39). CGA fall into the latter category; they are esters of *trans*-cinnamic acids (cinnamates) and quinic acid (28, 40). These polyphenols are abundant in coffee, with the main subclasses including caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloylquinic acids (FQA) which together constitute 98% of all CGA in coffee (**Table 2**) (28, 37, 40, 41). The predominant CGA in coffee is 5-caffeoylquinic acid (5-CQA) at 30-60% of total CGA (28, 42-45). CQA, diCQA and FQA are esters of

Table 1. Coffee and Risk of Type 2 Diabetes

Study	Population	Comparison	RR (95% CI)
MEDLINE published cohort studies (14)	193,473 males & females (15-98yrs)	0 or ≤2 vs. ≥6 or ≥7 cups/d	0.65 (0.54-0.78)
Nurses' Health Study II (46)	88,259 females (26-46yrs)	0 vs. 2-3 cups/d	0.58 (0.49-0.68)
25 Japanese communities (47)	17,413 males & females (40-65yrs)	0 vs. 3+ cups/d	0.58 (0.37-0.90)
E3N/EPIC cohort study	69,532 females (41-72yrs)	0 vs. ≥3 cups/d	0.73 (0.61-0.87)
ARIC Study (48)	5,414 males 6,790 females (45-64yrs)	0 vs. 4+ cups/d	0.77 (0.61-0.99) 0.83 (0.64-1.08)
Nurses' Health Study & Health Professionals' Follow-up Study (15)	41,934 males 84,276 females (30-75yrs)	0 vs. ≥6 cups/d	0.46 (0.26-0.82) 0.71 (0.56-0.89)
Iowa Women's Health Study (49)	28,812 females (postmenopausal)	0 vs. 6+ cups/d	0.78 (0.61-1.01)
Dutch prospective cohort study (50)	17,111 males & females (30-60yrs)	≤2 vs. ≥7 cups/d	0.50 (0.35-0.72)
Rancho Bernardo Study (35)	1,115 males & females (30-105yrs)	never vs. current	0.36 (0.19-0.68)
sNHANES-1 Epidemiological Follow-Up Study (51)	7,006 males & females (32-88yrs)	per 2 cups/d	0.87 (0.78-0.97)
Whitehall II cohort (52)	5,823 males & females (35-55yrs)	0 vs. 6+ cups/d	No significant risk reduction

Published studies of coffee consumption and the corresponding risk reduction (RR) of developing T2D. Adapted from (53).

Table 2. CGA Content of Coffee Beans

Compound	Green coffee beans (mg/g)	Roasted coffee beans (mg/g)
3-CQA	2.45 ± 0.18	0.78 ± 0.03
4-CQA	3.73 ± 0.24	0.86 ± 0.04
5-CQA	29.64 ± 2.08	1.42 ± 0.01
3,4-diCQA	0.85 ± 0.15	0.04 ± 0.00
3,5-diCQA	2.35 ± 0.30	0.02 ± 0.00
4,5-diCQA	1.43 ± 0.32	0.01 ± 0.00
4-FQA	0.15 ± 0.01	0.08 ± 0.00
5-FQA	1.04 ± 0.05	0.14 ± 0.00

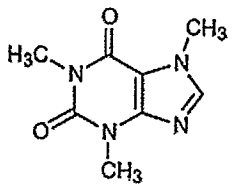
Chlorogenic acid content of green and roasted Columbian coffee beans. CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; FQA, feruloylquinic acid. Adapted from (54).

quinic acid and caffeic or ferulic acids respectively. Caffeic acid is the most abundant dietary phenolic acid while ferulic acid is found in high concentrations in grains; however neither are found as free acids but rather are present only in conjugated form (55, 56). While CGA are present in most fruits, they are largely concentrated near and in thepeel and so are less likely to be consumed (55, 57). Also, the actual amounts found are relatively small; for example, one would have to ingest ~1kg of apples to obtain an equivalent dose of CGA as 1 cup of coffee (57). Although blueberries are a good source of CQA, providing 0.5-2g/kg, the high rates of coffee consumption make it the most important dietary source of CGA (28).

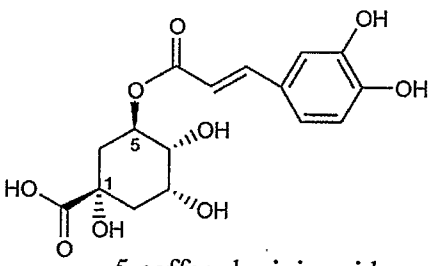
2.1.1 Content in Coffee

Green coffee beans contain 4-12% CGA, compared to caffeine at 1-2.5% (**Figure 1**) (40, 58). In fact, the concentrations of caffeine and CGA in green beans are linked, as some CGA forms 1:1 molecular complexes with caffeine (37). During roasting however, the CGA content is greatly reduced due to chemical changes associated with the high heat process. Depending on the desired degree of roast (dark, medium or light), green coffee beans are roasted at 210-240 °C for 10-15min (59). Initially isomerisation and partial hydrolysis occur, releasing quinic acid and the associated cinnamates (56). A portion of CGA are transformed to quinolactones (quinides) (40, 60), while some CGA may get incorporated intact into melanoidins (61). Brewed coffee therefore contains 35-150mg CGA per 100mL, with slightly less found in equivalent volumes of decaffeinated and instant coffees (22, 28). CGA concentration also varies with the coffee species and geographical origin (58). There are two main commercial coffee species: *Coffea arabica*

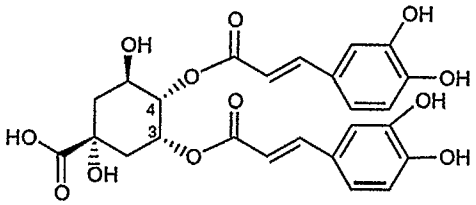
Figure 1. Structures of Coffee Components



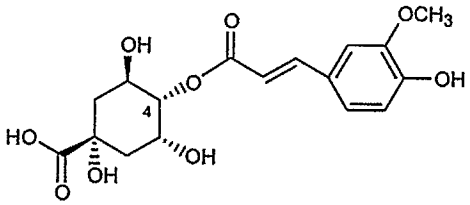
Caffeine



5-caffeoylquinic acid



3,4-dicaffeoylquinic acid



4-feruloylquinic acid

Chemical structures of selected coffee components, including caffeine and chlorogenic acid, adapted from (62).

(arabica) and *Coffea canephora* (robusta); in general, robustas have higher CGA (and caffeine) concentration than arabicas (43). During green coffee bean roasting, hydrolysis, oxidation and polymerisation all lead to substantial decreases in CGA, therefore light roasts have higher amounts than dark roasts (63). The CGA metabolites, mainly caffeic and quinic acids, are undetectable in brewed coffee (64).

2.1.2 Absorption

Controversy exists over the extent to which CGA are absorbed. It is likely that while some CGA is absorbed intact, the rest undergoes hydrolysis with subsequent absorption of the metabolites. To date, plasma analysis after coffee or CGA ingestion has detected only low levels of intact 5-CQA (42, 45, 65-67) or none at all (64, 68). Urinary excretion studies rarely recover intact CGA, and then only minor (<1%) amounts, but do identify many metabolites (39, 45, 65, 69, 70). All studies reference used high performance liquid chromatography (HPLC), which is the standard method of detection for CGA (22, 71, 72). However, Manach et al. (73) have suggested sample preparation with this technique may cause CGA degradation, resulting in little or no detection; this possibility was also noted by others (42). Interestingly, both caffeic acid and ferulic acid are absorbed well from the stomach and small intestine in animal models, appearing rapidly in both plasma and urine (56, 67, 74-77).

CGA is stable in artificial gastric juice (39, 78) and some CGA is absorbed intact from the stomach (79). Olthof and colleagues (70) determined that up to 33% of CGA is absorbed in human subjects without a colon, indicating some absorption occurs in the small intestine. This may involve absorption of the intact compound and/or metabolites.

Enzymatic hydrolysis of CGA was not found in either small intestine, liver or plasma extracts (80). In contrast however, differentiated Caco-2 (human small intestine epithelial) cells displayed esterase activity when incubated with hydroxycinnamates (81). Using the same cell model, paracellular diffusion of intact 5-CQA was demonstrated (82). Additionally, measurable amounts of caffeic acid have been found in the stomach and small intestine of CGA-fed rats, which suggests some hydrolysis occurs early in the GI tract (79). Timing of CGA analysis compared to administration may factor into conflicting results as 43% of orally administered CGA was recovered intact from the small intestine of rats 1h after ingestion, and 5% still remained 5h later (68). In humans maximum concentration of CGA appears ~2.25h after ingestion with colonic metabolites appearing 8-10h after ingestion, some peaking at 6h (42). (83, 84). Method of administration matters for appearance of compounds as gastric emptying from a meal differs from that of a liquid dose (85).

The colon has been identified as the main site of CGA metabolite absorption, following bacterial esterification (65, 80, 86). Extensive digestive activity occurs in the colon by gut microflora, and faecal extracts contain an esterase capable of hydrolysing ferulic acid esters (80, 87, 88). Indeed, several species of gut bacteria have been identified with this ability (89). As microbiota differs from person to person and can be altered by diet and in turn can alter body composition, individual differences are expected (90-92). Therefore any unabsorbed intact CGA remaining is hydrolysed in the colon, initially into the main metabolites of caffeic, ferulic and quinic acid (85). Further degradation then occurs, as these metabolites undergo several reactions to form benzoic acid which is absorbed and ultimately excreted as hippuric acid in urine (86). Caffeic and

ferulic acids and their derivatives can be absorbed from the colon, and are mainly found in conjugated forms in plasma (39, 64, 93). The most common conjugation processes are sulfation and glucuronidation which occur in the small intestine and the liver (39, 94). Given that at least some intact CGA circulates in the bloodstream, both CGA and its metabolites may have physiological effects throughout the body.

2.1.3 Antioxidant Capacity

Antioxidants combat free radicals that are formed naturally during body processes (95, 96). Free radicals, such as superoxide and the hydroxyl ion, can damage the body in a variety of ways including accelerating the formation of advanced glycation end products which are involved in both macro- and microvascular complications (97, 98). If levels of antioxidants are not sufficient to manage free radical formation, oxidative stress occurs (96, 97). It has been suggested that metabolic disorders, including T2D, result from oxidative stress (95, 97-99). People with T2D appear to have higher levels of free radicals than healthy populations (100). To combat this imbalance, need for increased dietary antioxidant intake is indicated. Due to low rates of consumption of fruits and vegetables however, the majority of dietary antioxidants actually come from coffee (101, 102).

Coffee contains several constituents that are demonstrated antioxidants, including CGA, melanoidins, lignans, vitamin E and Maillard reaction products (29, 41, 103, 104). The main antioxidants in coffee are 5-CQA with the metabolites caffeic and ferulic acid retaining antioxidant capabilities, though to a lesser extent (41, 70). CGA have been

shown to be involved in free radical scavenging, metal chelation, low density lipoprotein oxidation protection, and DNA damage inhibition (105-108).

2.1.4 CGA In Vivo

As discussed above, at least some CGA is absorbed intact in the body. There appears to be systemic effects of CGA ingestion, including a role in weight loss. Drinking CGA-enriched coffee 5 times per day allowed overweight human participants to lose an average of 5.4±0.6 kg in 12 weeks (109). Significant weight loss was also noted in subjects consuming green coffee extract daily compared to a control beverage (110). The authors suggested that there was reduced glucose uptake from the small intestine and reduced glucose release from the liver. Although CGA have also been shown to alter blood mineral concentration (108, 111), reduce cholesterol levels and lower both plasma and liver triglycerides (31), their role in blood glucose control is focused on here. While CGA does not increase glucose uptake into skeletal muscle (112), there is evidence for actions of CGA in the small intestine and the liver.

2.1.4.1 Small Intestine

Glucose is absorbed in the small intestine via sodium (Na^+) dependent transport across brush border membranes. Welsch et al (30) isolated rat membrane vesicles *in vitro* and found glucose uptake to be reduced by 80%, 38% and 35% with 1mM 5-CQA, ferulic acid and caffeic acid respectively. Other phenolic compounds have likewise been shown to inhibit glucose uptake (113). Possible mechanisms of action for the reduced uptake by CGA have been suggested: dissipation of the Na^+ electrochemical gradient;

binding of sulfhydryl groups on glucose transporters resulting in conformational changes; glucose transporter inhibition due to the phenolic hydroxyl group and lastly, α-glucosidase (maltase) inhibition (30, 114).

Several studies have found glucose ingested with coffee or CGA (24, 109, 115-117) to reduce blood glucose concentration rise. Together, these studies suggest that CGA in coffee inhibits glucose absorption thereby attenuating insulin secretion. This would effectively lower the glycemic response of foods ingested with coffee (118).

2.1.4.2 Liver

CGA has been shown to inhibit hepatic glucose output *in vivo*. CGA reduces the activity of glucose-6-phosphate (G-6-P) translocase 1, a critical transporter involved in glucose synthesis in the liver (25, 119, 120). Chronically infusing 5-CQA into rats for 3 weeks significantly lowered their peak blood glucose concentration during an oral glucose tolerance test, suggesting an interaction of CGA and the liver (31). Similarly, rats fed a diet supplemented with instant caffeinated and decaffeinated coffee for 3 and 12 months had slightly lower fasting blood glucose concentrations compared to those fed standard chow (121). Chronic decaffeinated coffee ingestion increased whole-body glucose clearance during a hyperinsulinemic-euglycemic clamp compared to placebo (116). Bassoli and colleagues (117) confirmed the ability of CGA to significantly reduce glucose output from the liver *in vivo*, but did not find this carried over in a rat model. Indeed, it appeared as though there was a lack of CGA uptake into liver cells, however this was determined with liver perfusion experiments (117). Together, these studies

indicate that chronic CGA administration results in reduced blood glucose, and suggests that coffee ingestion may have a significant effect over time.

2.2 Blood Glucose Management

2.2.1 Intestinal Glucose Absorption

Carbohydrate digestion begins in the mouth with salivary amylase breaking internal α-1,4 links of polysaccharides; this process is continued by pancreatic α-amylase in the lumen of the small intestine, producing oligo-, tri- and disaccharides (122, 123). Digestion continues at the brush border membranes, where hydrolysis of disaccharides occurs on the upper villi of enterocytes with disaccharidases cleaving the terminal α-1,4 linkages (124). Thus lactase breaks down lactose; maltase cleaves maltose and maltotriose, and the sucrase-isomaltase complex splits sucrose and α-limit dextrins, respectively (124). This enzymatic process results in the appearance of monosaccharides: glucose, galactose and fructose.

Glucose absorption occurs via uptake into enterocytes, mainly through sodium glucose co-transporter 1 (SGLT-1) (125). A sodium-potassium pump (Na^+/K^+-ATPase) found basolaterally on the absorptive cell drives the electrochemical gradient (125). Thus one mole of glucose is transported with two moles of sodium. Intercellular glucose has three fates: some may be used for intracellular fuel; some is converted to glucose-6-phosphate which gets incorporated into vesicles where dephosphorylation occurs prior to exocytosis into blood and the remainder is directly transported into blood through the facilitated glucose transporter GLUT-2 (125). SGLT-1 also transports galactose and the glucose analogue 3-O-methylglucose, but not 2-deoxy-D-glucose (which is transported

by glucose transport facilitators GLUT-1 and GLUT-2) (125, 126). SGLT-1 has been shown to be inhibited by several polyphenols, including epicatechin gallate and epigallocatechin gallate, 2 flavanols found in green tea (127).

2.2.2 Insulin Control

Glucose ingestion and absorption results in a rise in circulating blood glucose, which stimulates insulin release. The endocrine pancreas synthesizes and secretes insulin from β-cells (128, 129). Insulin promotes glucose uptake into the liver for short-term storage as glycogen, and into tissues such as skeletal muscle for fuel (129). Simultaneously, insulin increases glucose uptake into adipose tissue for long-term storage as triglycerides (TG) while inhibiting lypolysis and free fatty acid (FFA) release (128).

The fuel source of tissue cells is constantly in flux depending on the plasma concentration of nutrients at a given point in time (129). When glucose is present in high concentrations, it is preferentially taken up by skeletal muscles and adipose tissue; when levels are low, FFAs are taken up instead (128). This process is mediated by insulin, which is responsible for recruiting the glucose receptor GLUT-4 to the surface of tissue cells (128). Therefore an increase in plasma glucose causes an increase in insulin, which works to lower blood glucose concentration by promoting glucose uptake into tissues. Insulin's main role is to reduce blood concentrations of both glucose and FFA.

2.2.3 Gastrointestinal Hormones

Circulating glucose is not the only trigger for insulin release. It was shown that a glucose dose given orally resulted in higher insulin response than if the same glucose

dose was administered intravenously (130, 131). This discrepancy was later found to be due to the release of gastrointestinal hormones in response to nutrient ingestion that enhance insulin secretion (131, 132). GIP (initially named "gastric inhibitory polypeptide") and GLP-1 are the two identified hormones secreted in the small intestine in response to food which fit the criteria (131). Known as incretins, they are responsible for 50-70% of insulin response and act in a synergistic manner (24, 131, 133). Both are in the glucagon-secretin family (134). GIP is a 42 amino acid peptide derived from a 153 (human) or 144 (rodent) amino acid proGIP precursor (135-137), whereas GLP-1 is a 30 amino acid hormone cleaved from the proglucagon gene (131, 138). Tissue specific posttranslational processing of proglucagon is required for GLP-1 production, as several other peptides including glucagon, GLP-2 and oxyntomodulin are liberated depending on cleavage sites (131, 139). Both GLP-1 and GIP precursors are processed in the intestine by tissue-specific prohormone convertases (136, 139).

Although receptors for both of these hormones are found throughout the body, including the gut, brain and other tissues, their main target is the pancreas (131, 140-142). Incretins stimulate insulin release by targeting specific G-protein coupled receptors on pancreatic beta-cells, resulting in production of cyclic AMP and multiple downstream targets that work together to produce insulin (25, 131, 143, 144). Additionally, *in vitro* and *in vivo* rat models suggest GLP-1 and GIP have a protective effect on beta cells by inhibiting apoptosis and promoting proliferation (25, 131). Loss of β-cell function leads to insufficient insulin release and impaired glucose tolerance, therefore maintaining β-cell mass may protect against development of T2D (145). Indeed, GLP-1 and GLP-1 agonists are being studied as anti-diabetic agents (25, 131).

2.2.3.1 GIP

GIP is a gut hormone secreted by K-cells, which are dispersed throughout the intestine but proximally concentrated in the jejunum (131, 146). K-cells can be stimulated both indirectly and directly through neural, hormonal and nutrient regulation (147). Vagal innervation stimulates GIP secretion in humans but not rats; β-adrenergic stimulation increases GIP release whereas α-adrenergic stimulation has the opposite effect (147-149). Evidence exists for paracrine inhibition of GIP secretion by somatostatin, a hormone released by D-cells; an incretin self-limiting feedback loop is also suggested as GIP itself promotes somatostatin secretion (147, 150, 151). GIP is released in response to the absorption of fat and glucose in humans as well as protein in rats (25, 146, 147, 152-154). Within 10-15min of oral ingestion of these nutrients, GIP reaches peak concentrations prior to their arrival in the gut indicating non-nutrient stimulation (133).

Active GIP [1-42 amide] released from K-cells is cleaved to GIP [3-42 amide] by dipeptidyl peptidase IV (DPP-IV). DPP-IV enzymatically cleaves off N-terminal amino acids (155). DPP-IV is found in numerous areas of the body, notably the intestinal brush-border membranes, as well as the kidney, liver, vascular endothelial cells and in plasma. Thus active GIP has a half-life of only 2min in rodents and 5-7min in humans (131, 156). The truncated GIP [3-42] does not stimulate insulin secretion and may in fact be a GIP receptor antagonist, albeit weakly (131, 146).

Basal circulating GIP is 10-20pmol/L, rising to 100-300pmol/L after meal ingestion (131, 139, 157). GIP seems to be unaffected in humans with T2D, with similar

secretion rates, circulating levels, and elimination although fasting levels are higher in obese compared to lean healthy humans (131, 144, 157).

2.2.3.2 GLP-1

GLP-1 is released from distally concentrated intestinal L-cells in response to presence of carbohydrates, fatty acids, essential amino acids or fiber (131). Of interest, recent evidence points to significant colocalization of GIP and GLP-1 in the mid-intestine (158). Like K-cells, L-cells are stimulated by several mechanisms. GLP-1 is secreted biphasically, with the first phase occurring 10-15min after meal ingestion and therefore likely to be due to neural or endocrine stimulation of the L-cells as nutrients would not have yet reached the distal gut (131). The second phase is 30-60min after food intake, likely due to direct nutrient stimulation (131). As with GIP, somatostatin works to reduce GLP-1 release while GIP itself increases intestinal proglucagon mRNA and GLP-1 secretion in rats but not humans (131, 134, 147).

There are two bioactive forms of GLP-1: GLP-1 [7-37] and GLP-1 [7-36] amide with other inactive forms, GLP-1 [1-37] and GLP-1 [1-36] amide, secreted simultaneously (131, 134, 140). Active GLP-1 is degraded by DPP-IV and has a shorter half-life than GIP of 2min in humans (131, 156, 159). Like GIP, the metabolites of active GLP-1, GLP-1 [9-36] amide and GLP-1 [9-37] are inactive and may also be GLP-1 receptor antagonists (139, 155, 160). Since DPP-IV is found in cell membranes on blood vessels and in plasma, the majority of intestinally-secreted active GLP-1 is inactivated in portal circulation (139, 161). In addition to its shared roles with GIP, GLP-1 inhibits

glucagon secretion from pancreatic alpha cells, reduces food intake and slows gastric emptying (139, 140, 162, 163).

Fasting plasma concentrations of GLP-1 are in the 5-10pmol/L range in humans, and tend to reach maximal levels of 30-40pmol/L postprandially (131, 157). These maximal levels are reduced in individuals who are obese or have T2D, yet excretion rates are unchanged (131, 157). This indicates the rate of GLP-1 secretion is probably lower in these populations, so it is physiologically desirable to increase GLP-1 in people with these conditions (131, 144, 164).

2.3 Background Summary

Several aspects of CGA have been explored in an attempt to link coffee ingestion with beneficial health effects, specifically blood glucose management. Although *in vitro* studies suggest CGA reduces glucose secretion from the liver via G-6-P modulation, *in vivo* studies have failed to confirm this. This may be due to the findings that only small amounts of CGA are absorbed intact. Additionally, although the antioxidant activity of CGA is well established, it is not clear that T2D results from oxidative stress. There is evidence for beneficial blood glucose effects from chronic CGA/coffee ingestion however. Weight loss is also demonstrated with coffee consumption, not all of which is attributable to caffeine. It then remains that the most likely acute role of CGA is that of attenuating blood glucose rise by inhibiting absorption of glucose along the small intestine. To date, only one study measured blood incretin levels along with glucose and insulin following coffee consumption. Results demonstrated that decaffeinated coffee ingestion reduced GIP and enhanced GLP-1 response compared to placebo (24). This

study aims to determine if CGA is responsible for the changes in incretin hormone levels and lower blood glucose rise seen with coffee consumption using both an *in vivo* and *in vitro* model.

2.4 Research Question

The objective of this study is to explore the mechanisms by which chlorogenic acid positively regulate blood glucose and insulin responsiveness. Previous studies have suggested three ways in which CGA may act: as an antioxidant, an inhibitor of hepatic glucose output and a suppressor of intestinal glucose absorption. This work aims to increase evidence for the latter by examining plasma glucose, insulin, NEFA and incretin hormone response to CGA ingestion in a rat model. Specifically, the research questions being addressed are: 1) does 5-CQA, compared to placebo, reduce glucose absorption and result in increased GLP-1 and decreased GIP response which alters insulin response and 2) does 5-CQA itself, or its main metabolites, alter GLP-1 secretion when glucose is present?

Although several studies have been done looking at CGA in rat models, they have measured blood glucose changes only; 1 using chronic intravenous administration (31), and 2 others using acute oral administration (68, 165). None of these studies measured insulin or incretin levels. One human study examined the GLP-1 and GIP concentration after decaffeinated coffee ingestion, and found significant changes with an OGTT that they attributed to CGA (24). Since there are thousands of bioactive compounds in coffee however, this causal relationship cannot be confirmed unless CGA is tested alone. To date, no studies have been done using a cross-over design to eliminate individual

response variation that appears to be prevalent (42). Elucidating the mechanism of CGA action is important to determine the most effective method of delivery, and this study aims to shed light on the role of 5-CQA in glucose absorption. If 5-CQA reduces glucose absorption and alters incretin response, it may be an effective treatment and/or preventative tool for T2D.

Chapter Three: CGA *In Vivo*

3.1 Introduction

Type 2 diabetes (T2D) is a serious disease rapidly rising in prevalence. Characterized by either inadequate insulin production or inability to utilize insulin produced, T2D results in elevated blood glucose levels (1). Epidemiological studies show decaffeinated and caffeinated coffee consumption to be correlated to reduced risk of T2D in a dose-dependent manner (13, 15, 16, 166). A systematic review by Van dam & Hu (14) found relative risk reductions of 0.65±0.12 (95% confidence interval) with ≥6 cups per day. Despite convincing epidemiological evidence, the component(s) of coffee responsible for these anti-diabetic effects is unclear. As caffeine is known to impair insulin sensitivity, a component of coffee other than caffeine is likely responsible (18, 19).

Chlorogenic acid (CGA) are the main antioxidants in brewed coffee, found in similar concentrations as caffeine (57). It is likely that these compounds are involved in mediating coffee's effects on blood glucose and insulin sensitivity as CGA have been found to reduce intestinal glucose absorption and inhibit hepatic glucose output *in vitro* (30, 31). The ability of CGA to impact glucose absorption along the gut depends on its presence in the intestinal tract. Indeed it appears that little CGA is absorbed intact from the stomach and small intestine, with the remainder undergoing hydrolysis and subsequent absorption of the metabolites in the colon (79, 85, 86). To date plasma and urine analysis after coffee or CGA ingestion has detected only very low levels of intact CGA (42, 65, 70) or none at all (79, 80). Timing of CGA analysis compared to administration appears to be a factor as 43% of orally administered CGA was recovered

intact from the small intestine of rats 1h after ingestion (68). Due to its prolonged presence in the intestine, it is worth exploring the ability of CGA to alter glucose absorption.

Oral intake of glucose results in the release of gastrointestinal hormones that enhance insulin secretion (130, 131). Known as incretins, GIP (initially named "gastric inhibitory polypeptide") and GLP-1 are responsible for 50-70% of insulin response and act in a synergistic manner (131, 133). Incretins stimulate insulin release by targeting specific G-protein coupled receptors on pancreatic beta-cells (25, 143, 144). GIP is secreted by K-cells dispersed throughout the intestine but proximally concentrated in the jejunum (131, 146). GIP is released in response to the absorption of fat and glucose in humans as well as protein in rats (147, 152-154). GLP-1 is secreted from distally concentrated intestinal L-cells in response to presence of carbohydrates, fatty acids, essential amino acids or fiber (131, 139). Although the two cell types are concentrated at opposite ends of the intestine, recent evidence points to significant co-localization of GIP and GLP-1 in the mid-intestine (158). Inhibited glucose absorption would be expected to lower rate of GIP secretion while increasing GLP-1 secretion due to sustained glucose presence in the intestine.

Previous *in vivo* studies have found glucose ingested with coffee (24, 116, 167), CGA (117), or CGA-enriched coffee (109) to reduce blood glucose concentration rise. One study found a reduction in peak blood glucose and plasma insulin of obese Zucker (*fa/fa*) rats after an oral glucose challenge following chronic intravenous administration of CGA (168), but no differences in blood glucose of human subjects were found after 4 months ingestion of CGA (169). No differences in blood glucose were found in fed

Wistar rats after intravenous injection of CGA, although an acute oral CGA dose reduced peak blood glucose following an oral glucose tolerance test (OGTT) (170). Only one study measured incretin levels, and found that decaffeinated coffee ingestion resulted in reduced GIP and enhanced GLP-1 response following an OGTT in human subjects (24). Since there are thousands of bioactive compounds in coffee however, CGA must be tested alone to establish a causal relationship. This is the first *in vivo* study to examine the acute effects of orally ingested CGA on blood glucose management in a conscious and unrestrained animal model. Additionally, ours is the first to use a cross-over design, as individual response variation appears to be prevalent (42).

3.2 Methods

3.2.1 Animals

Procedures were approved by the University of Calgary Animal Care and Use Committee (**Appendix A**) and abide by the Canadian Association for Laboratory Animal Science guidelines for animal experimentation. Male Sprague-Dawley rats (n=12) weighing 260-300g were housed individually in a temperature controlled room with a 12h light- dark cycle. Rats were given ad libitum access to drinking water and maintained on standard rat chow (5001 Laboratory Rodent Diet, Purina, Richmond, IN). Energy density of the diet (5001 Laboratory Rodent Diet, Purina, Richmond, IN) was 234g/kg of energy as protein, 45g/kg as fat and 499g/kg as carbohydrate. Experiments were performed on chronically catheterized, unrestrained, conscious rats. This model allows blood to be collected without increasing stress in the animal, as stress responses can alter insulin, glucose and free fatty acid concentrations (171-173).

3.2.2 Surgery

Rats underwent surgery to implant a catheter into the right carotid artery as described previously (174). Briefly, the surgical procedure involved anesthetising the rats with isoflurane, making a midline incision and isolating the left common carotid artery. The artery was catheterised with PE50 tubing under sterile conditions. The inserted catheter was flushed with heparinized saline (150U heparin/mL), then exteriorized and secured at the back of the neck where it was stopped with a stainless steel plug. Rats were allowed to recover for 4-5d prior to experiments, with weight gain monitored daily. Only those animals who regained weight to pre-surgery weight were used in experiments.

3.2.3 Dual Energy X-ray Absorptiometry

Body composition of recovered rats, including body fat and lean mass was determined 18h before start of experiments using duel energy x-ray absorptiometry (DEXA). This non-invasive procedure is the current gold standard for body composition analysis. Rats were lightly anesthetised with isoflurane for the duration of the scan, lasting ~2min.

3.2.4 Experimental Protocol

Experiments were performed in a cross-over design, with animals randomly being assigned to placebo or CGA treatment. Experiments were separated by a 3d washout period. A summary of the experimental protocol is shown in **Figure 2A**. Rats were fasted overnight (12h) with ad libitum access to water. Catheters were connected to additional PE50 tubing to allow for blood collection and were flushed with heparinized

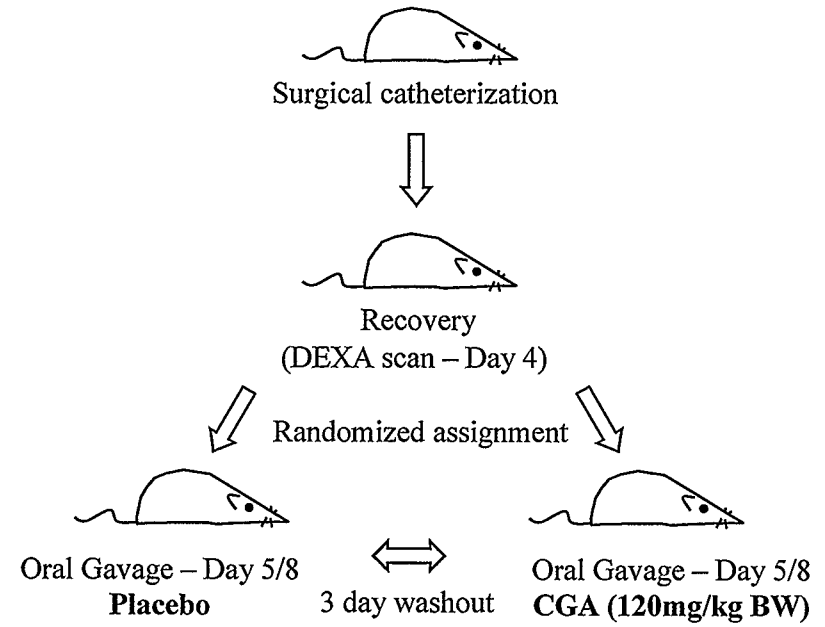
saline (10U heparin /mL) to prevent clotting. Baseline blood was collected ~5min before oral gavage. On each experiment day, rats were weighed and a meal dose (4g/kg body weight (BW)) calculated. The oral gavage consisted of 59% carbohydrate, 25% fat and 12% protein with water in a 1g dry food: 1.5mL water ratio; see **Appendix B** for gavage recipe. Acetaminophen (124mg/kg BW) was added to the mixture to track rate of gastric emptying (175, 176). Blood (~200-400ul) was then collected at 15, 30, 45, 60, 90, 120 and 180min after meal ingestion. For animals receiving CGA, the meal contained 120mg/kg BW chlorogenic acid hemihydrate (Sigma-Aldrich Co, Oakville, ON).

3.2.5 Plasma Measures

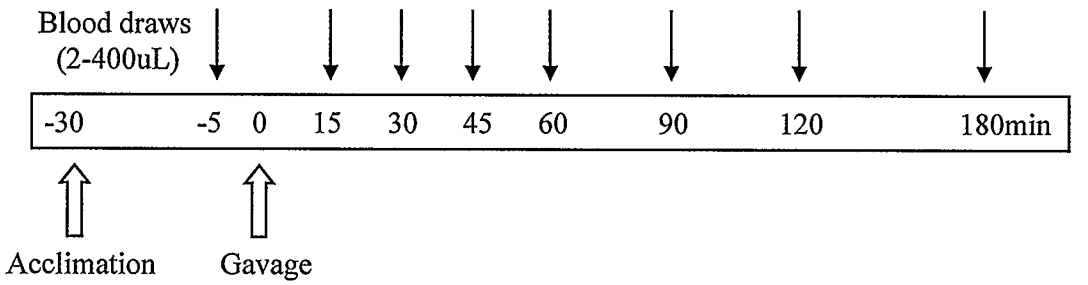
Blood glucose was measured with a whole blood glucose monitor (**Figure 2B**) (OneTouch Ultra 2, Milpitas, CA). Additionally, ~200-400ul blood was collected into a chilled EDTA-coated tube containing 1uL Diprotin A, a dipeptidyl peptidase-IV inhibitor to prevent GLP-1 and GIP degradation (177). Immediately after collection, plasma was separated by centrifugation and stored at -80°C until analysis. Analysis of insulin and GIP was carried out using the Rat Gut Hormone Panel LINCOplex Kit (Linco Research, St. Charles, MO). Samples of 12.5µL of plasma were analyzed using antibody-immobilized beads specific for these two hormones. Analysis of hormone concentration was determined using the Luminex^{100}. Active GLP-1 was quantified using the Glucagon-Like Peptide-1 (Active) ELISA kit (Linco Research, St. Charles, MO). Non-esterified free fatty acids (NEFA) were measured using HR Series NEFA-HR (2) kit (Wako, Chuo-Ku, Osaka). Acetaminophen levels were determined using Acetaminophen-SL Assay (Diagnostic Chemicals Limited, Charlottetown, PEI).

Figure 2. *In Vivo* Experimental Protocol

A



B



A) Cross-over study design. After recovery from surgery, rats' body composition was determined followed by 2 experimental days separated by a 3d washout period. **B)** Experiment day procedure. Baseline blood was drawn 5min prior to administration of oral gavage, with subsequent draws up to 180min thereafter.

3.2.6 Statistical Analysis

Differences between baseline body mass and blood glucose, GIP, GLP-1, insulin, NEFA and acetaminophen for the two treatment conditions were determined (SigmaStat for Windows Version 3.5, San Jose, CA). Area under the curve (AUC) values were calculated using the trapezoidal method. Comparison of results for individual time points and AUC were done using paired *t* tests following a Kolmogorov-Smirnov normality test. Differences with $P<0.05$ were considered significant. Values are expressed as means ± SEM.

3.3 Results

3.3.1 Baseline Measurements

Baseline blood glucose, plasma insulin, GIP, GLP-1 and NEFA are shown in **Table 3.** There were no significant differences between either the CGA or placebo groups, or between the values for each of the experiment days. DEXA analysis showed average body fat to be 8.12±0.62% among the rats. There was no significant correlation between percent body fat and fasting glucose, insulin, GIP, GLP-1 or NEFA (data not shown).

3.3.2 Blood Glucose

As seen in **Figure 3**, blood glucose rapidly increased from fasting levels in both placebo and CGA treatments following gavage, and concentrations were significantly higher at 15min in both groups ($p<0.001$). However, the rise in blood glucose concentration was attenuated following administration of CGA compared to placebo. There was a

Table 3. Baseline Measurements of Experimental Animals

	Placebo	CGA
Weight (g)	266.8 ± 4.9	264.9 ± 4.7
Fasting NEFA (mmol/L)	0.65 ± 0.04	0.59 ± 0.04
Fasting glucose (mmol/L)	5.46 ± 0.24	5.18 ± 0.19
Fasting insulin (pg/mL)	347.5 ± 47.3	364.4 ± 73.4
Fasting GIP (pg/mL)	51.7 ± 8.3	60.0 ± 4.1
Fasting GLP-1 (pmol/L)	6.06 ± 1.02	5.64 ± 0.93

Baseline characteristics of experimental animals following overnight (12h) fast. All blood samples were obtained by the arterial blood catheter. Data represent means ± SEM.

significantly reduced blood glucose concentration at 60min ($p=0.024$) for rats given CGA treatment. Total area under the curve (AUC) was also significantly lower for rats fed CGA compared to placebo ($p=0.021$) (**Table 4**). Blood glucose did not return to baseline in either CGA or placebo treated rats 180min after meal gavage ($p<0.001$).

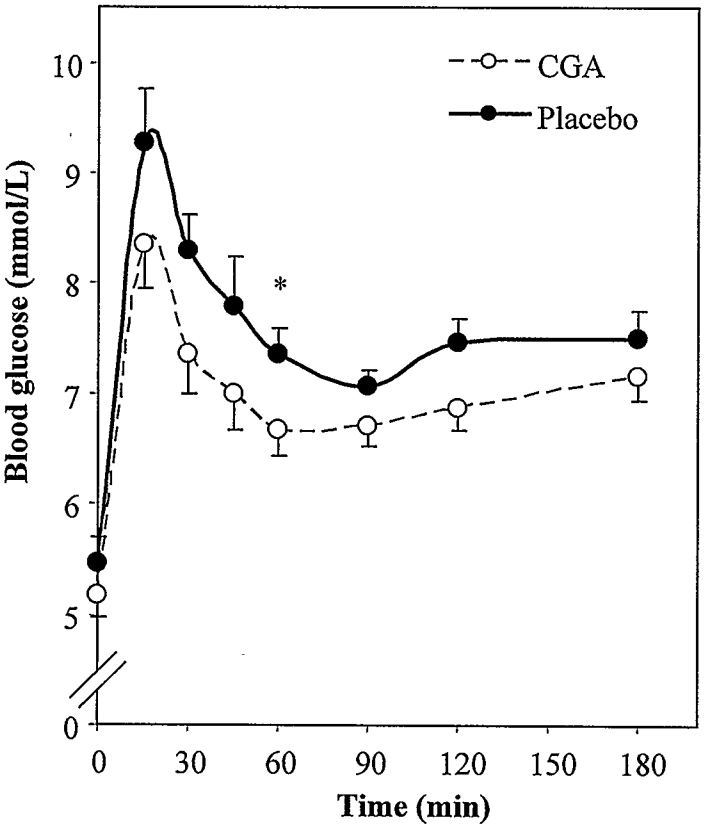
3.3.3 Incretins

Figure 4A demonstrates that plasma GIP increased from baseline following gavage in both groups, with a maximum response at 60min with placebo compared to 45min with CGA treatment. Ingesting CGA in a mixed-meal gavage resulted in significantly reduced GIP levels at both 30min ($p=0.036$) and 60min ($p=0.006$) respectively. Results demonstrated significantly lower AUC ($p=0.029$) for GIP over 180min compared to placebo **(Table 4)**. Plasma GIP concentrations returned to baseline after 180min in both groups. GLP-1 levels decreased shortly after meal ingestion in both groups, then increased again between 45-90min (**Figure 4C**). Despite attenuated secretion of GIP with CGA, there were no statistically significant differences in GLP-1 between groups, at any time point or for the AUC over 180min **(Table 4)**.

3.3.4 Insulin & Non-esterified Fatty Acids

Figure 4B shows that there was a rapid rise in plasma insulin levels in both CGA and placebo fed rats following mixed meal administration. Insulin concentration peaked at 15min in both treatment groups and dropped steadily thereafter. Although the rise in blood glucose was attenuated by CGA, no corresponding declines in insulin were observed between groups at any time point or for the AUC over 180min (**Table 4**).

Figure 3. Rat Postprandial Blood Glucose Response



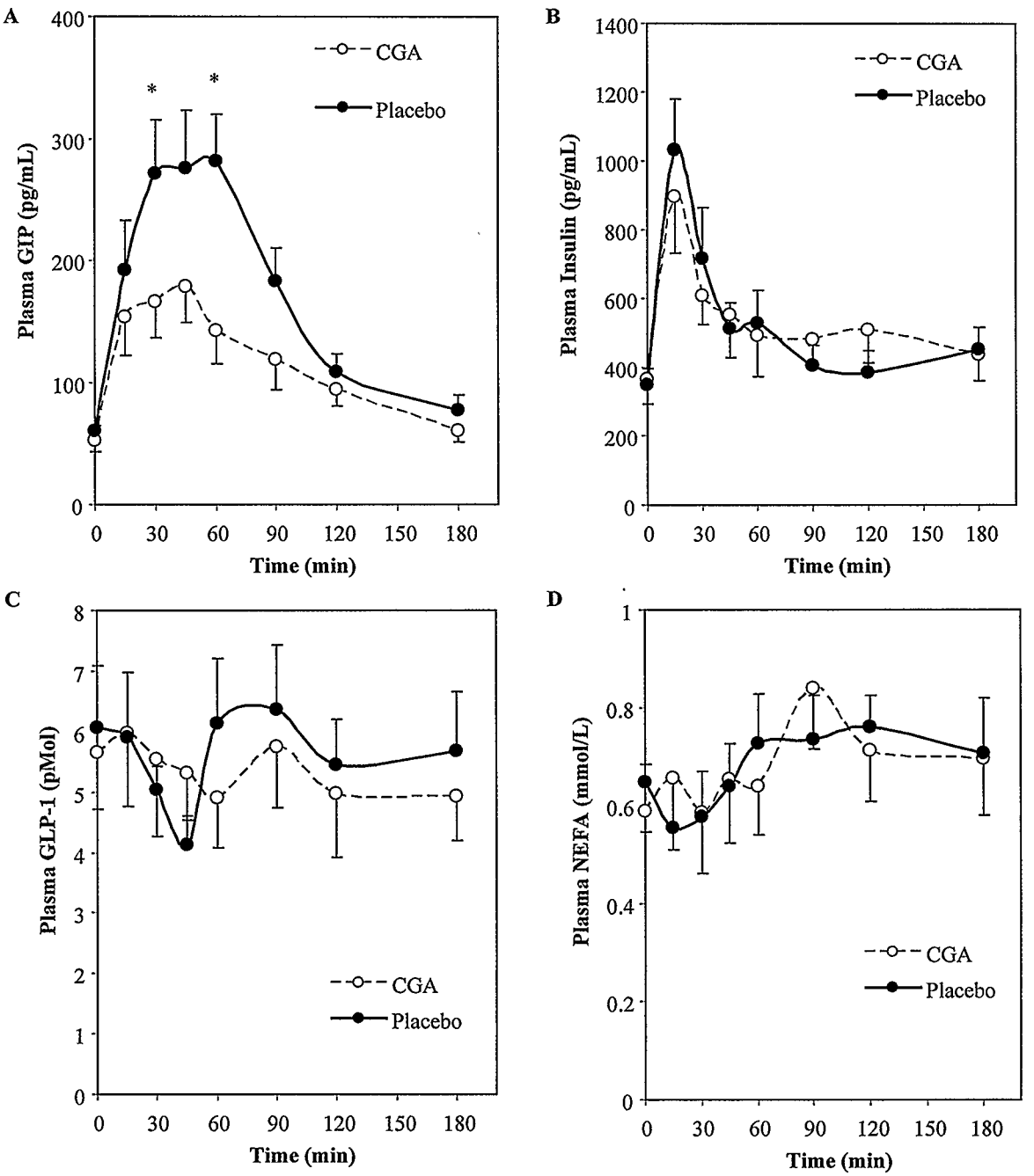
Blood glucose in response to mixed meal gavage with or without CGA. (**a**) Results represent the mean ± SEM for n=12 rats in each group. * Significant difference between treatment groups at t=60min (p=0.024).

Table 4. AUC for Rat Blood Measurements per Treatment

Measurement (/3h)	**Placebo AUC**	**CGA AUC**	**p-value**
Blood glucose (mmol/L)	1360.5 ± 25.9	1255.6 ± 28.0*	0.021
Plasma insulin (pg/mL)	91298 ± 10308	94932 ± 15004	0.837
Plasma NEFA (mmol/L)	125.2 ± 11.3	125.3 ± 16.2	0.993
Plasma GIP (pg/mL)	30510 ± 3683	20678 ± 2694*	0.029
Plasma GLP-1 (pmol/L)	1015.0 ± 79.1	948.8 ± 119.3	0.641
Plasma Acetaminophen (μg/mL)	3400.5 429.1	3173.3 541.8	0.667

Area under the curve (AUC) values for blood glucose, plasma insulin, NEFA, GIP and GLP-1. * Indicates $p<0.05$ between treatments.

Figure 4. Rat Postprandial Plasma Responses over 180min



(**A**) Plasma GIP response to a mixed meal gavage with or without CGA. (**B**) Plasma insulin response to a mixed meal gavage with or without CGA. (**C**) Plasma GLP-1 response to a mixed meal gavage with or without CGA. (**D**) Plasma non-esterified fatty

response to a mixed meal gavage with or without CGA. (**D**) Plasma non-esterified fatty acid (NEFA) response to a mixed meal gavage with or without CGA. Results represent the mean ± SEM for n=12 rats in each group. *Indicates $p>0.05$ between treatment groups at a given time point.

Additionally, while glucose concentrations did not return to baseline even after 180min insulin levels levelled out 45min after meal ingestion. Somewhat surprisingly, non-esterified fatty acid (NEFA) concentration did not significantly decrease after oral gavage in either treatment group as seen in **Figure 4D**. In the fasted state, NEFA levels are elevated as glucose stores are depleted. Postprandially, endogenous fatty acids decrease as circulating blood glucose increases. Our results show that the appearance of non-esterified fatty acids were unaffected by CGA treatment (**Table 4**).

3.3.5 Gastric Emptying

Gastric emptying was assessed by the administration and subsequent appearance of acetaminophen. Plasma levels peaked 15-30min after meal intake and steadily decreased thereafter in both CGA and placebo treatment groups. While several factors can affect gastric emptying, including GLP-1 secretion, no significant differences in the concentration of plasma acetaminophen were observed over the 180min of study ($p=0.667$, data not shown). Thus any differences between treatment groups in the blood measures of interest can be attributed to the absence or presence of CGA rather than alterations in nutrient arrival in the gut.

3.4 Discussion

Although evidence points to a protective effect of coffee intake on development of T2D , the mechanism of action has not been clarified. The present study examined the role of CGA, the main polyphenol found in coffee, in altering glucose absorption and subsequent incretin response. There is an abundance of information showing CGA to

inhibit glucose uptake *in vitro*, however *in vivo* studies are lacking. Given this, the aim of the present study was to assess the administration of CGA on blood glucose management *in vivo*. Employing a conscious animal model, we show that oral CGA (120mg/kg) intake in combination with a mixed meal significantly attenuated the increases in blood glucose. Analysis of the gut peptides, GIP and GLP-1 showed GIP release to be significantly reduced with CGA consumption, while GLP-1 secretion was not altered. Acetaminophen administration indicated that these changes were not due to differences in gastric emptying. Although glucose and GIP levels were reduced with CGA treatment, neither insulin nor NEFA levels were modified.

We found acute CGA consumption to reduce the rate of blood glucose absorption in the presence of a standardized meal dose, as evidenced by a slower rise in blood glucose concentration compared to placebo treatment. The glycemic index (GI) measures the AUC for the blood glucose response over 2-3h compared following consumption of a given food, compared to a to a white bread standard (178). A low glycemic index (GI) diet may be recommended to reduce postprandial glucose and insulin (179). Thus our results show that CGA effectively lowered the glycemic index of the meal administered. A low glycemic diet is one of the recommendations to prevent or treat T2D as it helps prevent chronic hyperglycemia (10, 179). Thus it is logical that chronic intake of CGA in the form of coffee may result in less rapid absorption of glucose when consumed with or close to a meal, reducing the risk of T2D.

The attenuated blood glucose rise observed in our study with CGA administration indicates a reduction of the rate of glucose absorption, which likely accounts for the significantly reduced GIP secretion observed. Johnston et al (24) also noted a decrease in

GIP following decaffeinated coffee consumption by human subjects compared to control. GIP is released in the proximal intestine in response to the rate of absorption of nutrients. In rodents, carbohydrates are a more effective stimulator than other nutrients therefore an alteration in glucose absorption is expected to have the greatest impact on GIP secretion (131). In contrast, no increase in GLP-1 secretion with CGA administration was observed. GLP-1 secretion occurs more distally in gut than GIP, concentrated in the ileum and colon (131). Although reduced glucose absorption would allow for increased glucose presence in the lumen, nearly all sugars are absorbed in the upper small intestine (126). Thus an increase in glucose presence in the duodenum and jejunum may not cause a corresponding increase in GLP-1. Likewise, in rats, GIP stimulates GLP-1 secretion (134). Thus the lower GIP found with CGA treatment could counteract any increase in GLP-1. Additionally, GLP-1 secretion can be inhibited by insulin as well as factors not measured in this study including somatostatin, a peptide hormone, and galanin, a neuropeptide (131).

The difference in blood glucose could not be explained by changes in insulin levels, as insulin was not significantly different after CGA treatment compared to placebo. No differences in insulin AUC were noted after caffeinated or decaffeinated coffee consumption compared to control beverage in healthy human subjects despite differences in GIP levels (24). Insulin is secreted by pancreatic β-cells in response to elevated blood glucose; incretins are responsible for 50-70% of the insulin response (131, 133). The relative contributions of GIP and GLP-1 on insulin stimulation are debated, although it is clear that they each contribute (180-182). GIP circulates in higher concentrations than GLP-1, yet GLP-1 appears to be a more potent stimulator (133, 183).

In this study GLP-1 secretions were not altered with CGA treatment however GIP was significantly reduced.

Gastric emptying was tracked to ensure rates of nutrient arrival in the gut were similar in both treatment groups. Data on polyphenolic intake and gastric emptying are scarce, with one study finding ferulic acid to increase rate of gastric emptying and gastrointestinal transit time (184). Acetaminophen administration is an indirect measure of gastric emptying as it is not absorbed significantly in the stomach but is rapidly absorbed in the small intestine (175, 176). Results show no significant difference in gastric emptying with CGA compared to placebo as measured by acetaminophen appearance. The rate of gastric emptying is controlled by multiple factors however, including GLP-1. Both GLP-1 (7-36 amide) and GLP-1 (7-37) dose-dependently inhibit gastric emptying (163). In this study no differences in GLP-1 secretion or gastric emptying were found, thus CGA does not appear to alter rate of gastric emptying when consumed with a meal dose.

This study employed a conscious, *in vivo*, unrestrained animal model to examine the effects of CGA on blood glucose management. This allowed for physiologically relevant data collection, as stress is known to alter measures of interest in this study, including insulin, glucose and free fatty acid concentrations (171-173). Additionally, we used a mixed-meal gavage as opposed to an oral glucose tolerance test as humans typically eat meals composed of several food groups, not single nutrients such as glucose. Although coffee consumption may not always be accompanied by a meal, CGA remains in the small intestine for up to 6h after administration (68). Although this is primarily a study looking at changes in blood glucose with CGA administration, the goal is to

uncover the mechanism(s) by which this compound may be protective against T2D. A dose comparable to that expected from usual coffee consumption in humans is 500-1000mg/day (57). For this project a dose of 120mg/kg was used to ensure enough of the compound was present to see an acute effect on glucose transport.

3.5 Conclusion

We found CGA to have a protective effect on blood glucose rise and to reduce GIP secretion, likely by slowing the rate of glucose absorption. Indeed, CGA may reduce the glycemic index of foods especially if ingested prior to or with a meal. Since coffee is the main source of CGA, this compound may explain the risk reduction for T2D seen in coffee drinkers. More research is needed however, particularly studies looking at blood glucose levels after caffeine with CGA to determine if the beneficial effects of CGA can outweigh the negative effects of caffeine on insulin sensitivity. Since CGA reduced blood glucose rise and altered GIP response, it may be an effective treatment and/or preventative tool for T2D.

Chapter Four: CGA *In Vitro*

4.1 Introduction

Chlorogenic acid (CGAs) are the predominant polyphenols present in brewed coffee, which is the main dietary source of CGA (57). CGA are esters of *trans*-cinnamic acids (cinnamates) and quinic acid; in coffee 5-caffeoylquinic acid (5-CQA) predominates at 36-60% of total CGA (28, 37, 40-44). CGA have been connected with a variety of health benefits as they demonstrate antioxidant, anticarcinogenic and antibacterial properties (41, 185, 186). As such, they have been suggested to play a major role in mediating the reduced risk of type 2 diabetes (T2D) seen in moderate to heavy coffee drinkers (25, 103).

The mechanism of action for CGA remains unclear, given that controversy exists over the extent and site of absorption due to limited recovery of intact CGA in plasma or urine (42, 45, 65). A plausible explanation for the benefits of CGA lies in their ability to alter intestinal glucose uptake. Welsch et al (30) showed caffeic, ferulic and 5-caffeoylquinic acid to all inhibit sodium-dependent glucose uptake in rat brush border membrane vesicles. At concentrations of 1mM, 5-CQA reduced glucose absorption by 80%; a 35% and 40% reduction was seen with caffeic acid and ferulic acid respectively. However Johnston et al (187) did not find such a relationship when using 10-fold lower concentrations of these and other phenolic acids in Caco-2 intestinal cells. The authors suggested the discrepancy may be due to differing ratios of the test agent to glucose.

Additionally, CGA may play a role in altering gut hormone response in the gut. Known as incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent

insulinotrophic polypeptide (GIP) are hormones intimately involved in insulin secretion (131). GIP is secreted from K cells in the proximal intestine in response to glucose absorption, while GLP-1 secretion is concentrated more distally from L cells in response to glucose presence (147). Human subjects ingesting either caffeinated or decaffeinated coffee experienced lower GIP concentrations over 3h compared to control beverage, while GLP-1 levels were elevated after decaffeinated coffee consumption (24).

The aim of this study was to clarify the ability of CGA to inhibit glucose absorption and thereby alter GLP-1 response *in vitro* using the human colon cell line NCI-H716, known to secrete GLP-1 in a regulated manner. Specifically, it was determined whether i) 5-CQA or 2 main coffee metabolites, caffeic and ferulic acid, interfere with GLP-1 secretion in the presence of glucose, and further whether ii) 5-CQA could influence GLP-1 secretion in the absence of glucose or if iii) 5-CQA could indeed alter glucose uptake in these human L-cells (**Table 5**).

4.2 Methods

4.2.1 Cell Model

The NCI-H716 human colon cell line was used for all three experiments. This enteroendocrine L-cell line is derived from a poorly differentiated human colon adenocarcinoma and has been shown to secrete GLP-1 in a regulated manner (188, 189). Methods were adapted from Reimer and colleagues (189). Briefly, identical cell culture conditions and GLP-1 secretion study techniques were utilised.

Table 5. *In Vitro* Experiment Summary

	Experimental conditions	**Glucose present**	**Outcome measured**
Experiment 1	5-CQA, caffeic acid, ferulic acid	Yes	GLP-1 secretion
Experiment 2	5-CQA	No	GLP-1 secretion
Experiment 3	5-CQA	Yes	Glucose uptake

Human colon NCI-H716 cells were incubated with varying concentrations of 5-caffeoylquinic acid (5-CQA) or coffee metabolites caffeic and ferulic acids in several conditions.

4.2.2 Cell Culture Conditions

Cells were grown in suspension in RPMI 1640 medium (Life Technologies, Inc) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc), 2mM L-glutamine (Life Technologies, Inc), 100 IU/mL penicillin (Life Technologies, Inc) & 100μg/mL streptomycin (Life Technologies, Inc). Media mixture was added every 3-4d until desired cell density reached, then every 7d for proliferation maintenance.

4.2.3 Secretion Studies

Two days prior to the experiments, $1x10^6$ cells were seeded into 12-well plates coated with Matrigel (Becton Dickinson and Co.) to induce endocrine differentiation for GLP-1 secretion (190). Media was simultaneously changed to low glucose DMEM (Life Technologies, Inc) supplemented with 10% FBS, 2mM L-glutamine, 100 IU/mL penicillin and 100μg/mL streptomycin as described above. For the secretion experiments, cells were washed 2 times and the differentiation media replaced with Krebs-Ringer bicarbonate (KRB) buffer containing 0.2% bovine serum albumin (BSA) (Serological Proteins, Inc). For experiments 1 and 2, the compound of interest (5-CQA, caffeic acid or ferulic acid) was added to the KRB buffer solution and the pH adjusted to 7.2 with NaOH. In experiment 1 cells were incubated in six 12-well plates with treatments in duplicate of control (no test agents, no glucose), positive control (2% meat hydrosylate) and i) 100 uM 5-CQA with various concentrations of glucose, ii) 5% glucose with various concentrations of 5-CQA, iii) 100 uM caffeic acid with various concentrations of glucose, iv) 5% glucose with various concentrations of caffeic acid v) 100 uM ferulic acid with various concentrations of glucose or vi) 5% glucose with

various concentrations of ferulic acid. For experiment 2, cells were incubated in two 12-well plates, either for 2h with varying concentrations (0-10mMol) of 5-CQA or for various amounts of time (15min increments up to 2h) with 1μMol 5-CQA. In both experiments 1 and 2, after the 2h incubation period supernatants and cells were collected separately. Supernatants were removed and 50ug/mL anti-protease phenylmethylsulfonyl fluoride (PMSF) (Sigma) with 2uL/mL DPP-IV inhibitor Diprotin A (Calbiochem) added to the collection to prevent GLP-1 degradation. Cells were scraped with lysis buffer containing both 50ug/mL PMSF and 2uL/mL Diprotin A. Both cells and supernatants were stored at -80°C until analysis.

4.2.4 Secretion Analysis

For experiment 1, GLP-1 concentration in both cells and supernatants was determined with GLP-1 (Active) ELISA (Linco Research, Inc, St Charles, MO). GLP-1 was measured using the GLP-1 (7-36) Active RIA kit (Linco Research, Inc, St Charles, MO) assay after 10-fold dilution of both cells and supernatants in experiment 2. The RIA analysis was done for the first experiment executed, experiment 2, as it was the technique of choice for Kristine Lee of Dr. Reimer's lab. The ELISA kit became available prior to experiment 1 and was chosen as it is more sensitive (2-200pM vs 10-500pM for the RIA) and safer as it does not use radioactivity in the method of detection.

4.2.5 Glucose Uptake

In experiment 3, $1x10^6$ cells were seeded into Matrigel-coated 12-well plates with low-glucose DMEM for endocrine differentiation 2d prior as described above. Methods

used to measure glucose uptake were then adapted from (187). Briefly, cells were incubated at 37° for 1h with low-glucose DMEM without FBS. Media was removed and cells were incubated at 37° with HEPES buffered salt solution (HeBSS) for 20min to halt cell glucose uptake. Finally, cells were incubated at room temperature for 12min in HeBSS alone (negative control) or containing 1mM glucose and 125kBq 2-[3H]deoxyglucose with and without (control) varying concentrations of 5-CQA; each treatment was done in duplicate. Supernatants were then discarded and cells washed 3x with 1mL ice-cold PBS. Cells were then incubated overnight at room temperature with 1mL NaOH (200mM). Glucose uptake was determined by measuring 0.5mL cell solution and 5mL scintillation fluid using the QuantaSmart (2.03) program.

4.2.6 Data Analysis

For all experiments, GLP-1 secretion analysis was measured in duplicate. Results are expressed as means ± SEM. Statistical analysis was done using the one-way ANOVA model (SigmaStat 3.5) to determine treatment differences. If differences in treatments were found, post-hoc analysis using the Student-Newman-Keuls method was used. Results were considered statistically significant if $p<0.05$.

4.3 Results

4.3.1 Experiment 1

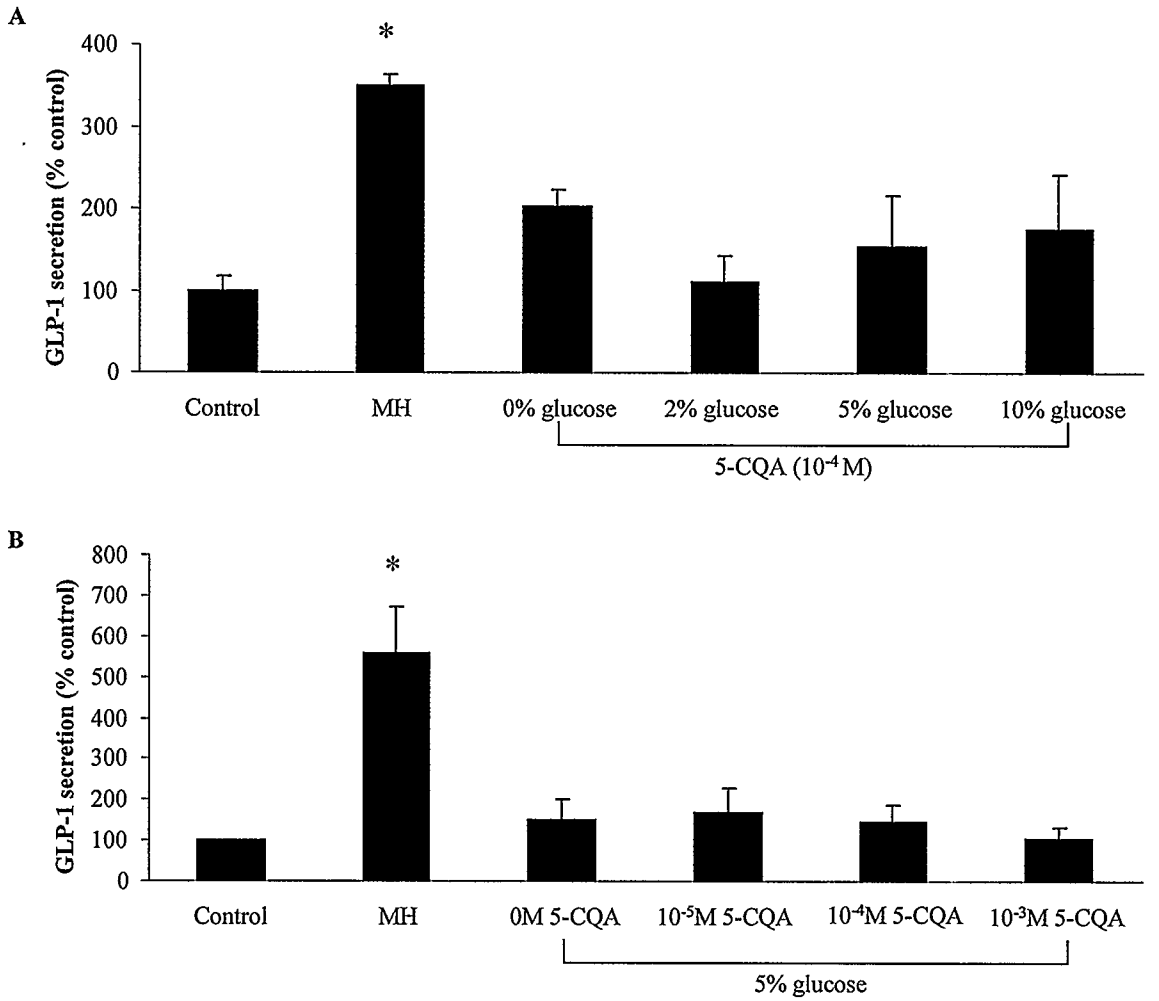
This experiment indirectly explored the ability of 5-CQA and two main coffee metabolites, caffeic and ferulic acid, to inhibit glucose uptake by measuring GLP-1 secretion. Meat hydrosylate (MH) was used as a positive control as it has previously

been shown to promote GLP-1 secretion (189). Our results corroborate this finding, as MH significantly increased GLP-1 secretion compared to control in 5 of 6 experiments. Results show 5-caffeoylquinic acid to have no effect on GLP-1 secretion, regardless of concentration tested or amount of glucose present (**Fig 5 A&B**). There was significantly increased GLP-1 secretion with 10^{-4}M caffeic acid at the highest concentration of glucose tested, but no other significant differences were noted with this polyphenol (**Fig 6 A&B**). Similarly 10^{-4}M ferulic acid with 10% glucose resulted in increased GLP-1 secretion compared to 0%, 2% and 5% glucose but not control or MH treatment (**Fig 7A**). There were no significant differences seen among cells incubated with varying ferulic acid concentrations with 5% glucose (**Fig 7B**). Therefore cells incubated with the intact ester of caffeic acid and quinic acid demonstrated no alteration in GLP-1 secretion, even among differing glucose concentrations, while cells co-incubated with 10% glucose and the coffee metabolites caffeic or ferulic acid increased GLP-1 secretion.

4.3.2 Experiment 2

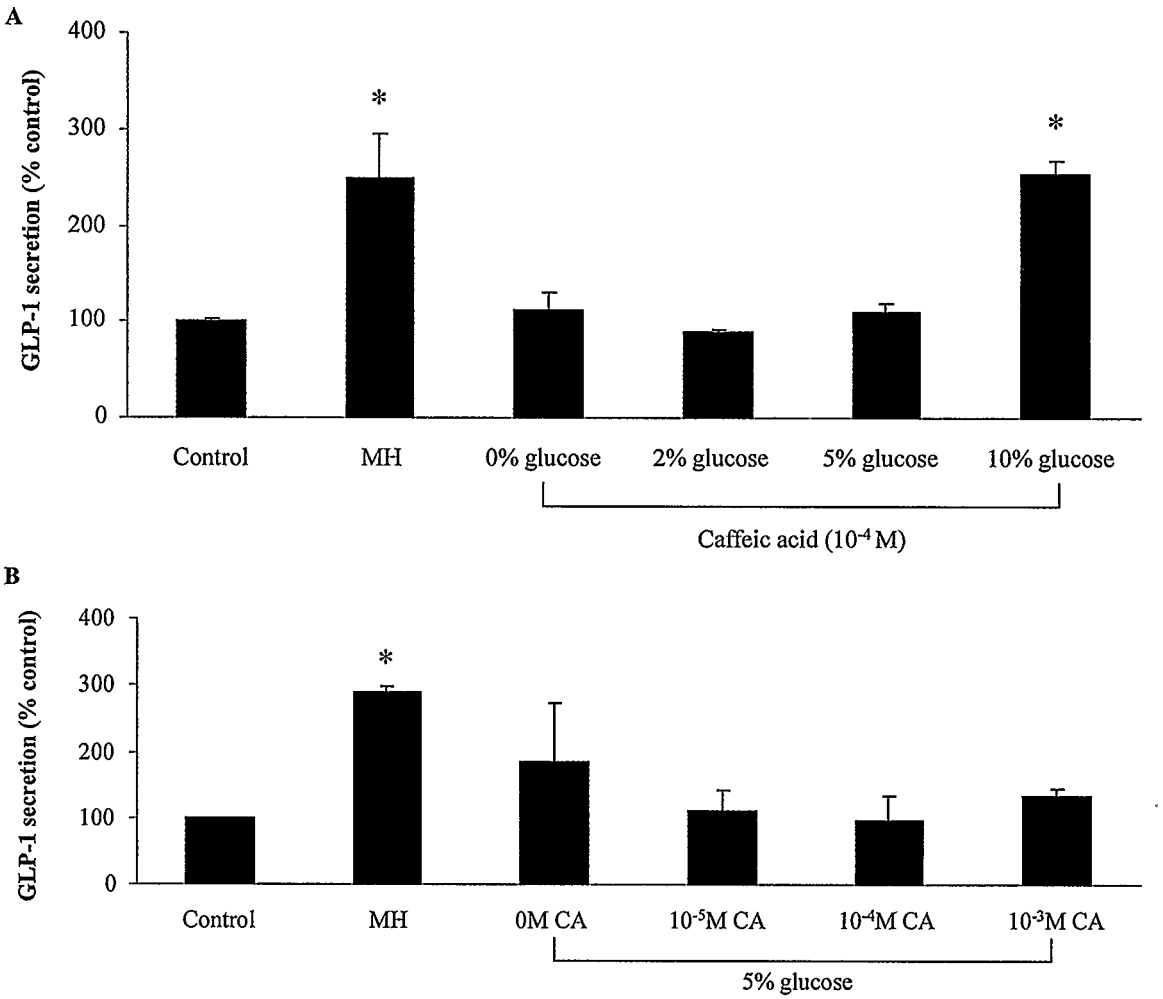
Human colon cells known to express GLP-1 were incubated with 5-CQA in varying concentrations for 2h and with 5-CQA (10^{-6}Mol) for varying times up to 2h (**Fig 8**). Results showed no significant difference between GLP-1 secretion for cells incubated for varying times with 10^{-6}Mol 5-CQA. For cells incubated for 2h with varying concentrations of 5-CQA however, GLP-1 secretion was significantly reduced with the two highest concentrations of 5-CQA (**Fig 9**). This may have been due to cell death, although pH was adjusted to 7.2 prior to incubation. Thus 5-caffeoylquinic acid does not alter GLP-1 secretion in human colonic cells in the absence of glucose.

Figure 5. Combined Effects of 5-CQA and Glucose on GLP-1 Secretion



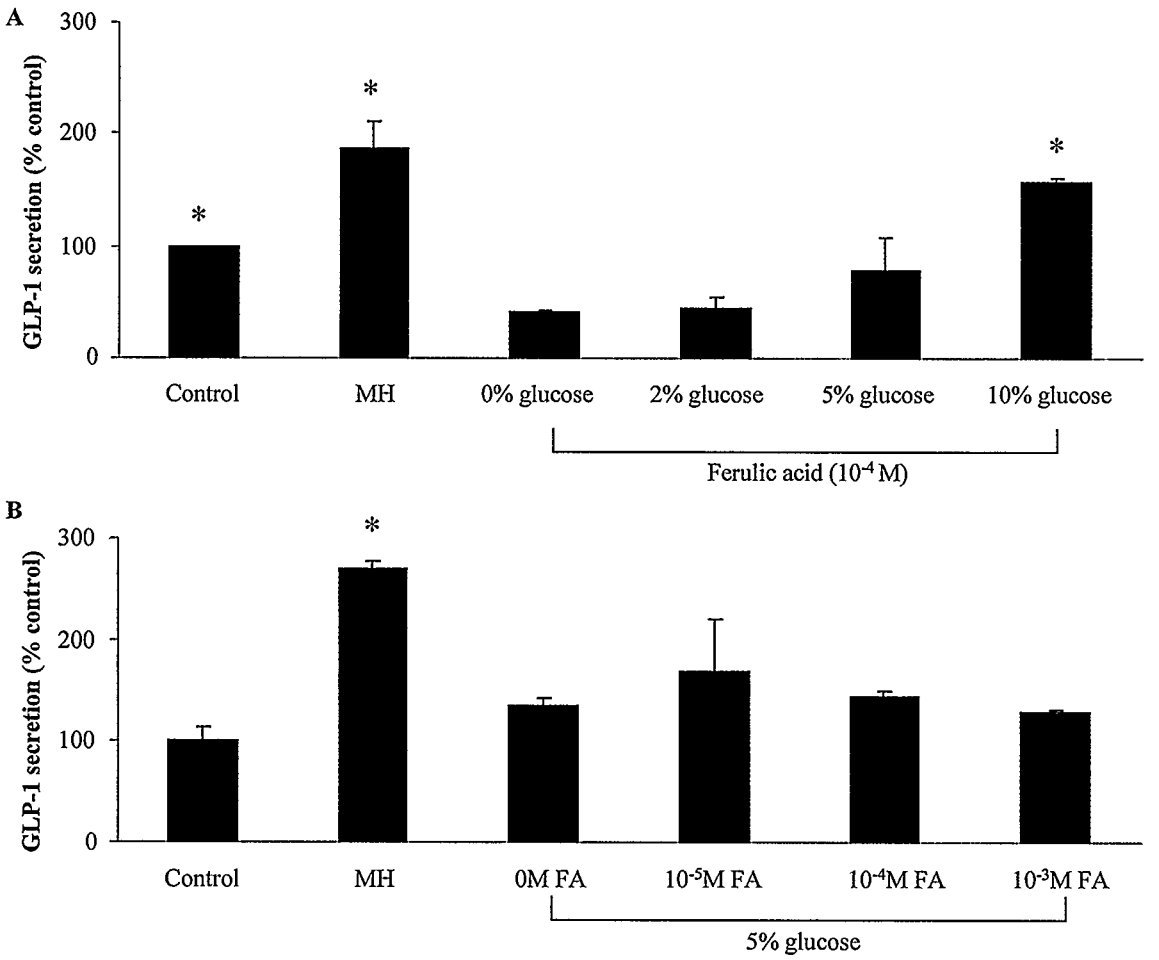
Experiment 1. **A.** Cells incubated with 10^{-4}M 5-CQA and varying concentrations of glucose for 2h. **B.** Cells incubated with 5% glucose and varying concentrations of 5-CQA for 2h. Values shown are means ± SEM expressed as a percentage of control (0Mol 5-CQA, 0% glucose). MH=Meat hydrosylate. * Indicates $p<0.05$ vs. other treatment groups.

Figure 6. Combined Effects of Caffeic Acid and Glucose on GLP-1 Secretion



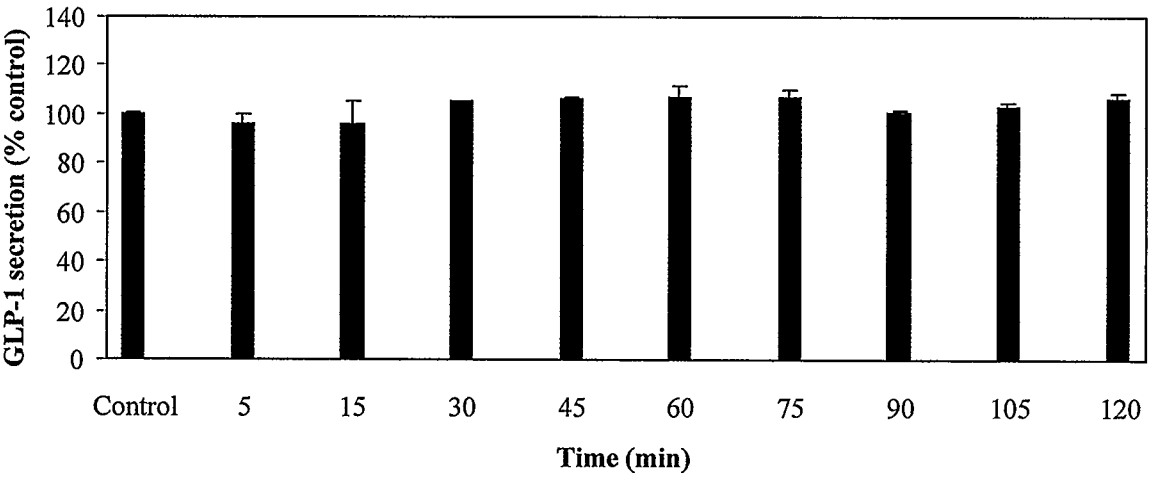
Experiment 1. **A.** Cells incubated with 10^{-4}M caffeic acid and varying concentrations of glucose for 2h. **B.** Cells incubated with 5% glucose and varying concentrations of caffeic acid for 2h. Values shown are means ± SEM expressed as a percentage of control (0Mol caffeic acid, 0% glucose). MH, meat hydrosylate. CA, caffeic acid. * Indicates $p<0.05$ vs. other treatment groups.

Figure 7. Combined Effects of Ferulic Acid and Glucose on GLP-1 Secretion



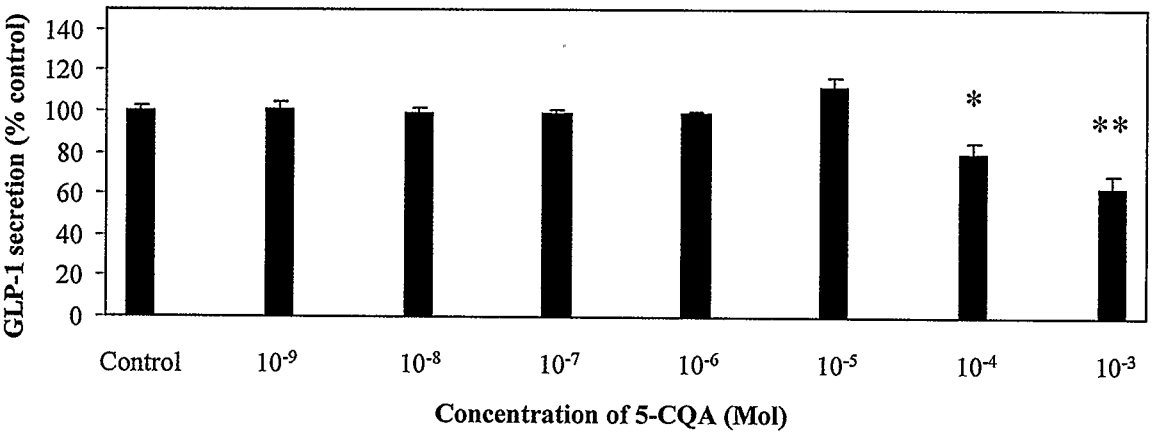
Experiment 1. **A.** Cells incubated with 10^{-4}M ferulic acid and varying concentrations of glucose for 2h. **B.** Cells incubated with 5% glucose and varying concentrations of ferulic acid for 2h. Values shown are means ± SEM expressed as a percentage of control (0Mol ferulic acid, 0% glucose). MH, meat hydrosylate. FA, ferulic acid. * Indicates $p<0.05$ vs. other treatment groups.

Figure 8. GLP-1 Response of Cells Incubated with 5-CQA over Time



Experiment 2. Incubation with 10^{-6}Mol 5-CQA for varying time periods up to 2h. Values shown are means ± SD expressed as a percentage of control (2h incubation without 5-CQA). No statistical difference was found among treatment groups (p=0.074).

Figure 9. Effect of 5-CQA Concentration on GLP-1 Secretion



Experiment 2. Incubation with varying concentrations of 5-CQA for 2h. Values shown are means ± SD expressed as a percentage of control (0Mol 5-CQA).

* Indicates $p<0.05$ from all other treatment levels ($p\leq0.030$); **$p<0.001$ from all other treatment groups.

4.3.3 Experiment 3

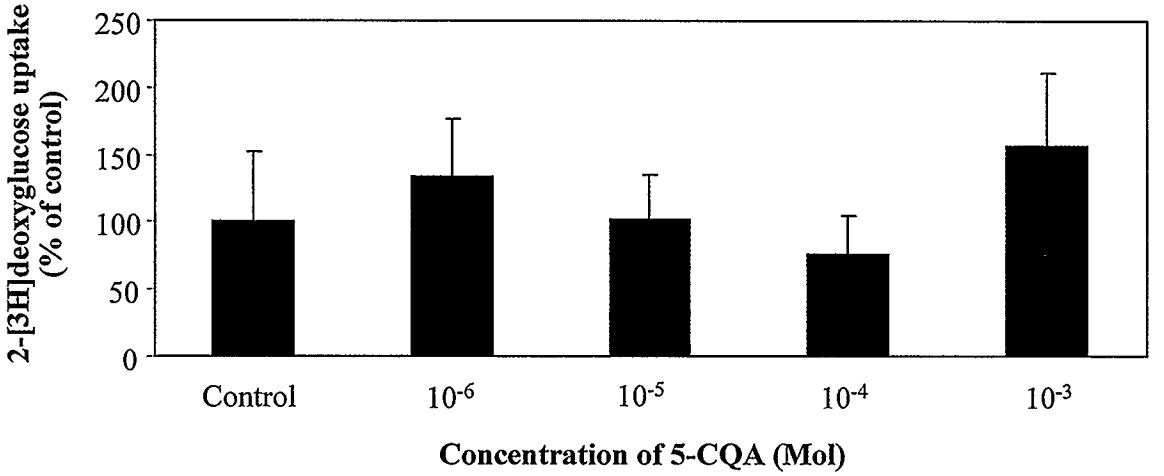
2-D-[3H]deoxyglucose was used to measure glucose uptake in NCI-H716 cells treated with various concentrations of 5-CQA. No significant difference of glucose uptake was found among treatment groups compared to control (**Fig 10**).

4.4 Discussion

In this project, alteration of glucose uptake and GLP-1 secretion was explored using the NCI-H716 human colon cell line in a variety of conditions. Based on the work of others, it was expected that CGA and 2 coffee metabolites would inhibit glucose uptake resulting in elevated glucose concentrations surrounding the cells and therefore increased GLP-1 secretion. In cells incubated with phenols, glucose uptake was directly measured as was GLP-1 secretion in the absence of glucose.

In experiment 1, cells incubated with the intact ester of caffeic acid and quinic acid (5-CQA) showed no alteration in GLP-1 secretion, even among differing glucose concentrations. In contrast, cells co-incubated with 10% glucose and the coffee metabolites caffeic or ferulic acids were found to increase GLP-1 secretion. GLP-1 is secreted from the NCI-H716 cell line in response to glucose presence: addition of 3% and 10% glucose resulted in approximately 1.5 and 2.7 fold increase in GLP-1 secretion compared to control (191). Thus if the polyphenolic compounds studied reduced glucose uptake, more glucose would be expected to remain in the cell solution, resulting in increased GLP-1 secretion. The results indicate that at the concentrations studied, intact chlorogenic acid is unable to alter glucose uptake in GLP-1 secreting cells. While Welsch et al (30) found a significant decrease of glucose uptake with 10^{-3}M 5-CQA, they used

Figure 10. Glucose Uptake in Cells in Response to 5-CQA Concentration



Experiment 3. 2-D-[3H]deoxyglucose uptake. Values shown are means ± SEM expressed as a percentage of control (0Mol 5-CQA). No statistical difference was found between groups (p=0.471). 5-CQA, 5-caffeoylquinic acid.

rat membrane vesicles which contain absorptive as well as enteroendocrine cells. Thus it may be that 5-CQA affects glucose uptake in absorptive cells rather than the GLP-1 secreting L-cells, although phenols did not alter glucose uptake in Caco-2 cells which express glucose transport proteins (187). Likewise, CGA had no effect on glucose uptake in muscle cells (112). Thus future glucose uptake studies may require absorptive and enteroendocrine cells to measure corresponding incretin release.

Experiment 1 found caffeic and ferulic acid increased GLP-1 secretion at high glucose concentrations; thus hydrolysis of CGA may be necessary to produce metabolites which have this ability. As glucose uptake was not measured directly in this study it is unclear whether the presence of the metabolites or, more likely, simply the higher concentration of glucose was responsible for the increased GLP-1 secretion. Further research involving incubation of this cell line with 10% glucose both with and without phenols would clarify the role of each. Additionally, although brewed coffee does not contain free phenolic acids, hydrolysis of CGA occurs resulting in caffeic and ferulic acid present in the intestinal tract (64).

In experiment 2, NCI-H716 cells were incubated in a buffer solution with 10^{-6}Mol 5-CQA for various amounts of time up to 2h or with varying concentrations of 5-CQA for 2h. Results demonstrated that at the 2 highest concentrations of 5-CQA tested, GLP-1 secretion was lower compared to control. It could be that cell viability was reduced however, since total GLP-1 was lower in these 2 treatment conditions (data not shown). No other significant differences were noted. Thus 5-CQA alone is not capable of increasing GLP-1 secretion in this human colon cell model, which lends evidence towards an indirect relationship involving glucose uptake.

In experiment 3, a small pilot study was run to determine if glucose uptake could be directly measured in the HCI-H716 cell line using 2-D-[3H]deoxyglucose. While no significant differences in glucose uptake were found among treatment groups of various concentrations of 5-CQA compared to control, no conclusions should be formed from this initial finding. The standard deviations are quite large within each treatment; possible reasons for this include that the cells may not have been incubated long enough with the treatment solutions to uncover such a difference, or cell viability within each well may have differed significantly. Repeating the experiment in triplicate may give better results. It is clear however that glucose uptake can be measured in the NCI-H716 cell line and that more work is required to optimize conditions.

4.5 Conclusion

Chlorogenic acid does not increase GLP-1 secretion in human colonic cells in the absence of glucose, nor does it appear to have an effect when cells are co-incubated with varying amounts of glucose. Concentration of 5-CQA does not alter the release of GLP-1 either, regardless of glucose presence or absence. The 2 coffee metabolites studied, caffeic and ferulic acid, did increase GLP-1 secretion but only in conditions of high (10%) glucose. These results indicate that the increased GLP-1 secretion is attributable to the glucose itself and not inhibition of glucose uptake as glucose presence is known to increase GLP-1 secretion. It may be that enteroendocrine cells alone are not an adequate model to study glucose uptake and corresponding incretin release, as presence of absorptive cells may also be required. Further evidence for this is the finding that glucose uptake in NCI-H716 cells is not altered by CGA presence, as measured by 2-D-

[3H]deoxyglucose. Thus mechanisms of CGA's effect on GLP-1 secretion remain to be determined and more research is required.

Chapter Five: General Discussion and Conclusion

5.1 Discussion

5.1.1 Introduction

Type 2 diabetes (T2D) prelevance is increasing worldwide, with a variety of methods being developed or improved to combat this alarming trend. Currently T2D is managed through lifestyle intervention and/or pharmaceuticals but the disease tends to progress and there is no true cure (1, 7). Thus effective antihyperglycaemic agents are important in reducing the prevalence and severity of this disease. Following epidemiological studies showing a dose-dependent relationship between increased coffee consumption and decreased development of T2D, chlorogenic acid (CGAs) have been examined for their blood glucose lowering ability (14, 16, 192). This study was designed to explore the role CGAs have on reducing intestinal glucose absorption using two models: an *in vivo* Sprague-Dawley rat model and a series of *in vitro* experiments using the NCI-H716 human colon cell line.

5.1.2 Study Strengths & Limitations

5.1.2.1 Acute vs. Chronic Studies

An acute feeding protocol was chosen for the Sprague-Dawley rat study as the primary goal was to discover whether glucose uptake was altered with oral CGA intake. Therefore using a one-time dose of CGA allowed for isolation of the effect of CGA taken with a meal without the potential complications of habituation or adaptation that may occur in a chronic study. Glucose is actively transported across the brush border

membrane by SGLT-1 (126). Although SGLT-1 can be upregulated by diet, especially high carbohydrate diet or increased glucose presence in the lumen, the process takes several days to occur thus an acute study does not need to take any intestinal adaptation into account (193-195). Additionally, the acute method enabled employment of a cross-over design so that age was not a confounding factor in the study, as glucose uptake decreases with age (196). This design also allowed for the surgical insertion of catheters for blood withdrawal. This method can only be employed for a short time period, as several rats' lines had clotted by the second experiment day (7d post-surgery). It would have been interesting to have added a "lunch" component to this study to determine if CGA, particularly the metabolites, played a role in blood glucose control given the finding that CGA remains in the intestinal tract for some time. There are of course benefits to a long term study. A longitudinal intervention would have been beneficial to enable comparisons to findings from human epidemiological studies. To equate results with these human studies however, CGA doses would have to be significantly lower than used in this study, and given throughout the day to be more representative of typical coffee consumption.

5.1.2.2 Animal vs. Human Studies

Sprague-Dawley rats are a standard laboratory rat with digestive systems similar to those of humans (197). They are a useful model for *in vivo* nutrition work as they are easily accessed, and allow for testing of compounds to be done in genetically and environmentally similar animals which reduces confounding factors compared to human subjects. Human research trials require recruiting and screening of volunteers to ensure

they meet study criteria, which can be both time consuming. Given this, it is logical to start with an animal model to demonstrate the relationship of interest and then progress to human subjects. There are drawbacks to using a rodent model for diet studies, as species differences do exist. For example, as previously mentioned, GIP promotes GLP-1 secretion in rats whereas this does not appear to occur in humans (134), thus results from animal studies may not be directly translatable. Glucose uptake results are expected to be transferrable between species however, as SGLT-1 is expressed similarly in both Sprague-Dawley rats and humans (198). Additionally, this study examined male rats only which may limit the extent to which extrapolations to females can be made.

5.1.2.3 Cell Culture

As with all cell culture work, results are indicative only of the specific environment in which the experiment occurs. This apparent disadvantage is also one of the great advantages of cell studies, as the conditions can be carefully controlled and altered as desired. For the experiments in this project, cells were incubated with known concentrations of substrates (glucose, CGA, caffeic and ferulic acid). In the *in vivo* animal study an exact dose of CGA was administered, but it would have been difficult to determine exactly where and when the compound reached L- or K-cells along the intestinal tract. Thus cell studies allow for limited but specific results which are especially useful for understanding mechanisms of action.

The NCI-H716 is an established cell line studied for its proven ability to secrete GLP-1 in a regulated manner. It is currently the only human L-cell line, although there are identified L-cell lines that release GLP-1 from other species, including the commonly

used murine GLUTag cells (199). Although NCI-H716 cells release GLP-1, proglucagon expression is aberrant which limits the analysis of GLP-1 secretion to the hormone presence alone, without the ability of measuring proglucagon gene transcription (200).

5.1.2.4 Plasma Analysis

Initially, a single kit (Rat Endocrine LINCO*plex*; Linco Research, St. Charles, MO) was chosen to analyse rat plasma for GIP, GLP-1 and insulin. This kit allows simultaneous quantification of multiple hormones with much less plasma than would be required for individual analysis, thus lowering the volume of blood drawn at each timepoint. Unfortunately, GLP-1 levels were too low to be detected with this kit, thus subsequent analysis with a separate assay was required (Glucagon-Like Peptide-1 (Active) ELISA kit; Linco Research, St. Charles, MO).

5.1.2.5 CGA vs. Coffee

Coffee contains thousands of constituents, of which CGA are only a portion. Additionally, 5-CQA is the only CGA commercially available and therefore the most studied. Thus actions of 5-CQA may not be representative of coffee, especially caffeinated coffee. If 5-CQA is the primary mediator of positive effects on blood glucose however, it is likely that administration of this isolated compound would be more effective than ingestion via coffee. This is due to the large variability of CGA content in brewed coffee depending on type of bean and degree of roast (60). Currently, a green coffee extract (Svetol) is available with high concentrations of CGA, but the exact

amounts and proportions of CGA likely differ from batch to batch due to the variability of green coffee bean contents. Additionally, whole CGA absorption is not complete, nor is the pathway understood, leaving the possibility that a dose-dependent effect on intact absorption exists.

5.1.2.6 Glucose Absorption vs. Appearance

The rat feeding study measured whole blood glucose from the general circulation. Although it is tempting to attribute changes in glucose appearance as measured by blood drawn from the implanted carotid catheter to alterations in glucose absorption, it may be that other factors, possibly unmeasured, are at play. Blood glucose appearance is attributable to absorption only for the first 30min post-ingestion in humans; after that peripheral metabolism plays a role in glucose concentration (24). The hyperinsulinemic-euglycemic clamp is the gold standard method of determining whole-body glucose uptake and its use would help clarify the role of glucose absorption versus blood concentration (26, 116).

5.1.3 Study Summary

Cell culture experiments using a human colon cell model demonstrated that 5-CQA alone is not capable of increasing GLP-1 secretion, findings which did not change when cells were co-incubated with 5-CQA and glucose in varying concentrations. GLP-1 secretion was only altered in cells incubated with high concentrations of glucose (10%), indicating glucose presence determined GLP-1 output and not presence of the phenolic compounds. This suggests glucose uptake rates may not have been sufficient to produce a

significant effect of phenolic inhibition. Although enzymes capable of separating esterified CGA have not been found in the upper GI tract, it is possible that metabolism of the intact compound is required to alter intestinal glucose uptake.

The *in vitro* finding that GLP-1 was not altered by CGA presence was also found in the *in vivo* study. Feeding 5-CQA acutely to rats resulted in significantly lower blood glucose over 3h. This was despite no differences noted in insulin, GLP-1 or NEFA concentrations, and was not due to changes in gastric emptying. This lends support for CGA as a contributor to the beneficial effects of coffee on blood glucose noted by previous studies.

The most significant finding of this work was the nearly 32% lower blood GIP concentration following CGA ingestion compared to placebo ($p=0.029$). Although reduced GIP secretion (implying reduced insulin stimulation) may seem counterintuitive for hyperglycemia prevention, GIP response is exaggerated in obesity and therefore inhibited release may in fact be desirable (201).

5.2 Future Research Directions

Human research is warranted, as very few studies with CGA have been done to date. Johnston et al (24) were the first to measure incretin levels after coffee consumption but they did not include a separate CGA group, thus results could not be specifically attributed to this compound. CGA-enhanced coffee was shown to lower blood glucose AUC following an OGTT compared to control, as well as reduce body weight compared to regular coffee after 12wk (109). Significant weight loss compared to controls was also noted in subjects consuming 400mg green coffee extract for 60d however no blood

measurements were collected (110). Recently, a human cross-over trial showed an acute dose of 5-CQA, but not decaffeinated coffee, to significantly lower blood glucose and insulin 15min following an OGTT compared to control (202). Given the significant differences in both glucose and GIP in our animal study and Johnston et al's (24) human study, additional human research is warranted. Because acute studies have shown small but significant effects of coffee and/or CGA on blood glucose, a longitudinal study with CGA alone would be beneficial.

As noted in Chapter 4, utilising HCI-H716 cells in monoculture may not allow for sufficient glucose absorption differences as it is not an absorptive cell line. GLP-1 is secreted from the NCI-H716 cell line in response to glucose presence (191). Welsch et al (30) found a significant decrease of glucose uptake with 10^{-3}M 5-CQA, however they used rat membrane vesicles which contain both absorptive and enteroendocrine cells. Phenols did not alter glucose uptake in Caco-2 cells which express glucose transport proteins (187), nor did CGA have an effect on glucose uptake in muscle cells (112). It may be that 5-CQA affects glucose uptake in absorptive cells rather than the GLP-1 secreting L-cells, thus co-culture would allow examination of CGA ability to alter glucose uptake and subsequent GLP-1 secretion. The most studied human absorptive enterocyte cell line is the Caco-2 cell line, which is derived from human colon adenocarcinoma (203). Caco-2 cells express SGLT-1 and several GLUTs, including GLUT-2, and are widely used in glucose uptake experiments (204-206). They are also the primary cell line used in *in vitro* polyphenol metabolism studies (187). It has previously been suggested that paracrine signalling from enteroendocrine cells to enterocytes occurs to regulate SGLT-1 expression, therefore presence of both cell types may be required to

see an effect of CGA-inhibited glucose uptake on GLP-1 secretion (207). Tang et al (208) successfully co-cultured NCI-H716 and Caco-2 cells in Caco-2 culture medium, thus their techniques could be adapted to repeat our experiments, especially experiments 1 and 3.

5.3 Conclusion

In conclusion, it appears that CGA, particularly 5-CQA, is effective in reducing blood glucose levels in rats when ingested orally. This is likely due to lower rates of glucose absorption in intestinal enterocytes which then alters incretin secretion, particularly GIP as it is released more proximally than GLP-1. Lower GIP may be desirable in attenuating hyperinsulinemia. In order to fully explore the relationship between glucose uptake and GLP-1 secretion, more research is required, possibly using co-culture of absorptive cells and enteroendocrine cells. Overall however, the effectiveness of 5-CQA as an antidiabetic agent is suggested and longitudinal human research is recommended.

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APPENDIX A: ETHICAL APPROVAL

UNIVERSITY OF CALGARY

Protocol BI 2008-42

Certification of Animal Protocol Approval

Applicant: Jane Shearer

Faculty/Department: Kinesiology

Project Title: The link between coffee and type 2 diabetes: chlorogenic acid and acute benefits on blood glucose management in male Sprague Dawley rats

Sponsoring Agency(s): UCRG Starter Grant

Effective: April 1, 2009 Expires: March 31, 2010

The Animal Care Committee,
having examined the animal care and treatment protocol,
approves the experimental procedures proposed and certifies
with the applicant that the care and treatment of animals
used will be in accordance with the principles
outlined in the most recent policies and
"Guide to the Care and Use of Experimental Animals"
By The Canadian Council on Animal Care.

Applicant — Date: July 2/09

Chair of Animal Care Committee or University Veterinarian — Date: July 2/09

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APPENDIX B: ORAL GAVAGE COMPOSITION

Ingredient	g/100g	kcal/g	kcal/100g	% kcal
Corn Starch	33.41	3.60	120.28	29.40
Sucrose	30.71	4.00	122.84	30.03
Protein (casein)	14.00	3.60	50.40	12.32
Lard	5.00	9.00	45.00	11.00
Soybean Oil	7.00	9.00	63.00	15.40
Dyetrose	5.00	0.00	0.00	0.00
AIN-93M Mineral Mix	3.50	0.84	2.94	0.72
AIN-93-VX Vitamin Mix	1.00	3.87	3.87	0.95
L-Cystine	0.18	4.00	0.72	0.18
Choline bitartrate	0.20	0.00	0.00	0.00
Totals	100.00		409.05	100.00

Macronutrient summary

Carbohydrate (59%)
- Corn Starch
- Sucrose

Protein (12%)
- Casein
- L-Cystine

Fat (25%)
- Soybean oil
- Lard