# Investigating the Mechanisms and Effectiveness of Common Buckwheat (*Fagopyrum esculentum* Moenech) for Acute Modulation of Glycemia

BY

Danielle Marie Stringer

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

# ABSTRACT

Type 2 diabetes (T2DM) is a chronic disease characterized by cellular insulin resistance and consequent disturbances in glucose metabolism. Long-term consumption of buckwheat has been previously shown to improve glycemia in individuals with T2DM; however, the underlying mechanisms as well as the contribution of improved acute glycemic responses have not been fully characterized. The current study used cell culture and clinical studies to investigate the mechanisms and effectiveness of common buckwheat for acute modulation of glucose metabolism and glycemia. Glucose uptake was inhibited in H4IIE cells treated with a buckwheat extract (BWE), an effect attributed to the actions of an unknown compound(s). Reduced glucose uptake and transepithelial glucose transport was also present in Caco2 colorectal adenocarcinoma cells and monolayers. The mechanism behind inhibited glucose uptake did not involve modulation of several signaling pathways regulating glucose metabolism, including p38 MAPK, p42/44 ERK, PI3Kγ, PKC, PKA, mTOR and AMPK. Interestingly, BWE treatment was associated with other effects on glucose metabolism, including elevated glucose production and levels of gluconeogenic enzymes. However, these effects were not mediated through the classical pathway of CREB activation involving cyclic AMP and PKA.

In a blinded, reference product-controlled study, consumption of a cracker product made from whole grain common buckwheat flour containing 50 grams of available carbohydrate was not associated with changes in post-prandial glucose or insulin concentrations in both healthy individuals and those with diet-controlled T2DM. However, consumption of buckwheat crackers was associated with changes in selected gastrointestinal satiety hormones. Interestingly, several significant correlations observed

between fasting concentrations and the overall post-prandial response of these hormones were affected by T2DM.

In conclusion, glucose-lowering effects of common buckwheat are not due to the actions of known bioactive compounds, and may involve direct inhibition of facilitative transporters by a novel compound. Although a buckwheat food product did not reduce post-prandial glycemia, identifying the compound responsible for inhibited glucose uptake will allow development of food products enriched with this compound, and may represent a more effective dietary approach to managing glycemia.

**ACKNOWLEDGEMENTS**

My sincerest thanks to my advisors, Dr. Peter Zahradka and Dr. Carla Taylor, for their support and encouragement throughout the course of my PhD work. This thesis is a product not only of my efforts, but also theirs and I hope they are as proud as I am of the work we accomplished together. I also thank them for the opportunity to work in their respective labs, which made me both a more capable scientist and a better person. Thanks are also due to the members of my committee, Dr. Michael Eskin and Dr. Chris Siow, as well as my external examiner Dr. Suresh Mathews. Your critical evaluation of this thesis was very much appreciated.

This project would not have been possible without the help of a number of people. In particular, I would like to thank Jordan Betteridge for his analytical work, Alysha Dudych for her perseverance on qPCR experiments and Heather Blewett, Angela Wilson, and Alanna Baldwin for their expertise and time spent on the human study. And of course, none of this work would have been possible without the efforts of Leslee Tworek, who not only assisted with experiments, but also kept the lab running.

I have been very fortunate to have worked in a lab with so many wonderful, fun, and enthusiastic people. Thank-you to the members of the Zahradka/Taylor labs for all of your support and for being great friends.

And finally, thank-you to my family and friends. Michael, your support and encouragement throughout this process has been amazing. Thank-you for keeping me going when all I wanted to do was quit. And to my mom and dad, who told me something very simple that stuck with me and got me through the rough times: don't worry about what other people are doing or thinking...just be yourself, work your hardest, and things will work out...maybe not how you expected, but they will work out for the best.

# TABLE OF CONTENTS

## LIST OF TABLES

**LIST OF FIGURES**

**ABBREVIATIONS**

| | |
|---|---|
| 2-DOG | 2-deoxy-D-glucose |
| AMPK | AMP-activated protein kinase |
| ANOVA | Analysis of variance |
| AS160 | Akt substrate of 160 kD |
| BSA | bovine serum albumin |
| BSA-TBST | bovine serum albumin in tris-buffered saline with tween |
| aPKC | atypical protein kinase C |
| BBM | brush border membrane |
| BLM | basolateral membrane |
| BMI | body mass index |
| BWC | buckwheat concentrate |
| BWE | buckwheat extract |
| cAMP | cyclic AMP |
| cDNA | complementary DNA |
| CI | confidence interval |
| CREB | cyclic AMP response element-binding protein |
| CTL | control |
| CVD | cardiovascular disease |
| D-CI | d-*chiro* inositiol |
| DM | diabetes mellitus |
| DMSO | dimethyl sulfoxide |
| ERK | extracellular regulated kinase |
| GIP | glucose-dependent insulinotropic peptide |
| GLP-1 | glucagon-like peptide-1 |

| | |
|---|---|
| GLUT | facilitative glucose transporter |
| GSK-3 | glycogen synthase kinase-3 |
| HDM | high density microsomal fraction |
| HOMA-IR | homeostasis model of assessment of insulin resistance |
| IPG | inositol phosphoglycan |
| IRS | insulin receptor substrate |
| LDM | low density microsomal fraction |
| MI | *myo*-inositol |
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| p38 MAPK | p38 mitogen activated protein kinase |
| p42/44 ERK | p42/44 extracellular regulated kinase |
| PBS | phosphate buffered saline |
| PEPCK | phosphoenolpyruvate carboxykinase |
| $PIP_3$ | phosphatidylinositol 3,4,5-triphosphate |
| $PIP_2$ | phosphatidylinositol 3,4-diphosphate |
| PIP | phosphatidylinositol 3-phosphate |
| PI3K | phosphatidylinositol 3-kinase |
| PLD | phospholipase D |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PKD | protein kinase D |
| PMA | phorbol-12-myristate-13-acetate |
| PP | pancreatic polypeptide |
| PSS | physiological salt solution |

| | |
|---|---|
| PYY | peptide YY |
| Rab-GAPs | Rab-GTPase-activating proteins |
| RQI | RNA quality index |
| SD | standard deviation |
| SDS-PAGE | sodium dodecylsulphate-polyacrylamide gel electrophoresis |
| SEM | standard error of the mean |
| SGLT | sodium-dependent glucose co-transporter |
| shRNA | short hairpin RNA |
| siRNA | small interfering RNA |
| Src | Src kinase |
| STZ | streptozotocin |
| T1DM | type 1 diabetes mellitus |
| T2DM | type 2 diabetes mellitus |
| TEER | transepilthelial electrical resistance |
| ZDF | Zucker diabetic fatty |

# Contribution Statement

The results in this thesis are presented in manuscript format (Chapters 4-7). I, Danielle Stringer, made the following contributions to the manuscripts:

Chapter 4:

- conducted Western blotting experiments to generate Figure 4.2 B, 4.5, and 4.6; performed Western blotting and glucose uptake experiments to generate Figure 4.7 D, E and F and performed glucose uptake experiments to generate Figure 4.8 C.

- Performed statistical analyses for the above-mentioned experiments

- interpreted the results of the above-mentioned experiments and wrote and edited portions of the manuscript based on these experiments

Chapter 5:

- Conducted experiments to generate all figures
- Performed statistical analyses for all experiments
- Interpreted results and wrote the manuscript

Chapter 6:

- Designed primers listed in Table 6.1 and qPCR experiments to generate data for Table 6.2
- Designed and performed experiments to generate data for Figure 6.1, 6.2 and 6.3
- Interpreted results and wrote the manuscript

Chapter 7:

- Prepared plasma samples from whole blood for analyses
- Performed all lab work, compiled results and conducted statistical analyses
- Interpreted results and wrote the manuscript

# Chapter 1: Overview

## 1.1 Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by insulin resistance and hyperglycemia. The incidence and prevalence of this chronic disease is increasing, despite a plethora of pharmacological agents available to combat hyperglycemia. As such, novel therapeutic options, including functional foods, are being sought and investigated to assist with glycemic management.

Buckwheat is a broad-leafed herbaceous annual plant that is genetically unrelated to wheat, but often classified and treated as a cereal due to common structural and chemical properties of its seeds [1]. Both epidemiological evidence and intervention studies suggest that consumption of buckwheat and food products made from buckwheat may be beneficial for glycemic management [2-4]; however, it is unknown if this positive relationship is due to acute effects on glucose metabolism, or involves a longer-term mechanism. Studies conducted in healthy mice and a rat model for type 1 diabetes suggest that acute exposure to buckwheat is sufficient to reduce post-prandial glycemia [5,6]. Buckwheat contains a number of phenolic compounds and other bioactive molecules known to affect signaling pathways regulating glucose metabolism and activity of glucose transporter proteins [7,8]; thus, the glucose-lowering effect of buckwheat observed in animals and humans might involve acute effects of these bioactives.

The overall objectives of the current study were to identify the signaling pathways controlling glucose metabolism that are affected by buckwheat and to determine if modulation of post-prandial glycemia after consumption of buckwheat contributes to the glucose-lowering properties observed in humans. Cell culture studies were employed to examine how bioactives in buckwheat affect signaling pathways regulating cellular

glucose uptake, glucose transport and glucose production in liver and intestinal cells, two key cell types involved in glucose uptake and metabolism. A randomized, reference-product controlled human study was designed to determine the acute effect of a food product made from buckwheat on the post-prandial glycemic and insulinemic response, as well as the post-prandial response of insulin-stimulating incretin hormones. The research in this thesis not only provides insight into the regulation of cellular glucose metabolism by bioactive compounds naturally present in buckwheat, but also evaluates the efficacy of a reasonably-achievable portion of buckwheat, incorporated into a food product, to reduce post-prandial glycemia in both healthy individuals and those with T2DM. The following section will review pertinent literature to provide context for the rationale behind the studies contained in this thesis.

## 1.2 Literature Cited

[1] The Canadian Special Crops Association. Buckwheat - Information. 2008. [On-line]. Available: www.specialcrops.mb.ca/crops/buckwheat.html. Accessed 25 September, 2012.

[2] Zhang, H.W., Zhang, Y.H., Lu, M.J., Tong, W.J., and Cao, G.W. (2007). Comparison of hypertension, dyslipidaemia and hyperglycaemia between buckwheat seed-consuming and non-consuming Mongolian-Chinese populations in Inner Mongolia, China. *Clin. Exp. Pharmacol. Physiol.* 34, 838-44.

[3] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia and hyperlipidemia. In: *Proceedings of the 5th International Symposium on Buckwheat*, Lin, R., Zhou, M., Tao, Y., Li, J., Zhang, Z., Eds.; Agriculture Publishing House: Beijing, China, 458-64.

[4] Wang, J., Liu, Z., Fu, X., and Run, M. (1992). A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: *Proceedings of the 5th International Symposium on Buckwheat,* Lin, R., Zhou, M., Tao, Y., Li, J., Zhang, Z., Eds.; Agriculture Publishing House: Beijing, China, 465-7.

[5] Hosaka, T., Nii, Y., Tomotake, H., Ito, T., Tamanaha, A., Yamasaka, Y., Sasaga, S., Edazawa, K., Tsutsumi, R., Shuto, E., Okahisa, N., Iwata, S., and Sakai, T. (2011). Extracts of common buckwheat bran prevent sucrose digestion. *J. Nutr. Sci. Vitaminol. (Tokyo)* 57, 441-5.

[6] Kawa, J.M., Taylor, C.G., and Przybylski, R. (2003). Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. *J. Agric. Food Chem.* 51, 7287-91.

[7] Johnston, K., Sharp, P., Clifford, M., and Morgan, L. (2005). Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett.* 579, 1653-7.

[8] Horbowicz, M. and Obendorf, R.L. (1994). Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. *Seed. Sci. Res* 4, 385-405.

# Chapter 2: Literature Review

## 2.1 Hexose Transporters

Glucose is a vital fuel source for the cell, providing energy through oxidative and non-oxidative generation of ATP. Effective energy harvesting from glucose is dependent on its entry into glycolysis in the cytosol; however, due to its polar nature, glucose does not readily diffuse across the plasma membrane and therefore requires the assistance of carrier molecules, known as transporters, to enter the cell. Glucose sources such as cellulose and starch are abundant in nature, and numerous organisms possess membrane proton symporters, substrate-binding transporters and group translocation systems to transport glucose [1]. Mammalian glucose transporters belong to one of two general categories: active transporters and facilitative transporters. Based on sequence comparisons, it is known that active and facilitative transporters originate from separate gene families, providing two distinct systems for glucose entry into the cell [2].

### *2.1.1 Active hexose transport: sodium-dependent glucose co-transporters*

The gene for the sodium-dependent D-glucose transporter was first cloned from the rabbit intestinal brush border membrane in 1987 [2], while cloning of the human counterpart, designated *SGLT1*, followed 2 years later [3]. Since sodium-dependent glucose uptake had been observed not only in the intestine but also in the kidney, cDNA encoding *SGLT1* from rabbit jejunum was used as a probe for Northern blot analysis of mRNA extracted from rabbit kidney cortex and medulla [4]. In addition to SGLT1, this probe hybridized to a smaller mRNA fragment detectable only in the renal cortex. These data were the first evidence for the existence of two SGLT transporters: SGLT-1, a high-affinity, low-capacity transporter expressed in the small intestine and straight tubule of the renal proximal tubule, as well as SGLT-2, a low-affinity, high-capacity transporter

expressed exclusively in the convoluted tubule of the renal proximal tubule [5,6]. More recently, SGLT-3, predominantly expressed in the kidney, intestine, liver and spleen [7], was discovered in humans and pigs [7,8]. In addition, six other SGLT genes expressed in the human and rabbit kidney have been identified, but the function of these orphan genes remains unknown [9].

SGLT transporters are characterized by 14 transmembrane segments, with extracellular C- and N-termini [10]. Glycosylation of an asparagine residue in the small exofacial loop connecting the fifth and sixth transmembrane segments is a post-translational modification essential for transporter function [11]. SGLTs use the transmembrane sodium electrochemical gradient, established by the basolateral $Na^+/K^+$ ATPase, to couple the energy derived from moving sodium down its concentration gradient to actively transport glucose into the cell. The stoichiometry of SGLT transporters is isoform-dependent, with SGLT-2 transporting 2 sodium ions for every glucose molecule and SGLT-1 and SGLT-3 transporting sodium and glucose in a 1:1 ratio [9,10,12]. Isoform differences also exist in the relative selectivity of substrate and affinity for transport. SGLT-1 will transport glucose and galactose as well as the synthetic sugar compounds α-methyl-D-glucopyranoside and 3-O-methyl-D-glucose with comparable affinity, while SGLT2 and SGLT-3 preferentially transport glucose over galactose or 3-O-methyl-D-glucose. In addition, the affinity of SGLT-1 for glucose is 10-fold higher than is the affinity of SGLT2 or SGLT-3 for glucose [10].

### *2.1.2 Facilitative hexose transport: GLUT transporters*

The mammalian SLC2A genes belong to a large gene superfamily whose protein products are involved in the transport of a variety of hexoses and other carbon compounds. The proteins coded by the SLC2A gene family are facilitative hexose transporters, and have been given the name “GLUT”. Hydropathy analysis of the first

identified GLUT transporter, GLUT1, revealed a protein structure containing 12 transmembrane α-helices that combined to form a central aqueous membrane channel for substrate transport [13]. This 12 transmembrane α-helical structure is characteristic of all GLUT transporters [14], as is the intracellular position of the C- and N-termini. Interaction of hydroxyl groups in hexoses with certain hydrophilic amino acids in the exofacial binding site of the transporter leads to a conformational change that pushes the substrate through the pore of the GLUT protein. Release of the hexose to the cytoplasm reverses the conformational change and returns the binding site to the outside of the cell [15].

To date, 14 functional GLUT isoforms have been identified and they are grouped into 3 classes based on sequence similarity [15,16]. Class I includes the 4 originally-identified transporters GLUT1, GLUT2, GLUT3, GLUT4 and the newly-identified GLUT14, which appears to have resulted from a gene duplication of GLUT3 with which it shares 93.5% of its nucleotide identity [17]. The class II transporters consist of GLUT5, GLUT7, GLUT9 and GLUT11; these isoforms can transport fructose in addition to glucose, but cannot transport galactose or the non-metabolizable glucose analogue 2-deoxyglucose. The class III transporters, GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13 are the least characterized; however, it is known that GLUT13, primarily expressed in the brain, is a transporter for myo-inositol and is the only GLUT protein that appears to function as a proton-coupled symporter [18]. The ability to transport glucose is the unifying link among these transporters; what sets them apart is their relative transport efficiencies and kinetics. Generally, most GLUT transporters are high-affinity, low-capacity transporters, with Michaelis-Menten constants ($K_M$; a measure of substrate concentration at which the reaction rate reaches half of its maximum value) ranging from as low as 0.3 mM for GLUT7 and GLUT9 to 6 mM for GLUT5 [18].

Due to the various roles of different tissues in glucose handling and metabolism, tissue-specific expression of glucose transporters is a product of tissue function and transporter kinetics. For example, GLUT3, the major neuronal glucose transporter expressed in both dendrites and axons, has a high affinity for glucose and the highest calculated turnover number of all GLUT isoforms, thus ensuring efficient glucose uptake by neurons [19]. GLUT2, unique among the facilitative hexose transporters due to its low-affinity, high-capacity for glucose (as indicated by the fact that it has the highest $K_M$ of all GLUT transporters), is expressed primarily in cells involved in glucose sensing such as hepatocytes and pancreatic β cells [20]. Hepatocytes, for example, must be able to respond to elevated plasma glucose concentrations typical of the post-prandial state require by continual net uptake of glucose. Conversely, in the post-absorptive or fasted state, hepatocytes must be able to release glucose produced from glycogenolysis or gluconeogenesis into the circulation despite relatively high steady-state glucose concentrations in the blood. Therefore, the low-affinity, high-capacity kinetics of GLUT2 allow the most effective glucose transport at high glucose concentrations, and the direction of glucose transport (i.e. influx vs. efflux) is determined by the relative intracellular versus extracellular glucose concentrations. Pancreatic β cells, which must be especially sensitive to changes in blood glucose concentrations to adequately adjust insulin secretion, must also express a transporter with a high $K_M$ (i.e. GLUT2) to prevent transporter saturation at high glucose concentrations, thus ensuring glucose flux is directly proportional to blood glucose concentrations.

Recently, the novel GLUT9 isoform has been postulated to contribute to glucose sensing by pancreatic β cells [21]. First cloned in 2000, GLUT9 has been characterized as a transporter for both glucose and fructose, showing significant homology to GLUT1 and GLUT5, with the highest expression in the kidney and liver [22]. Interestingly, GLUT9 displays considerably higher activity as a transporter for urate, an end product of

purine metabolism in humans, than it does for hexoses [23]. Based on several kinetic studies, GLUT9 has been proposed as a high capacity urate transporter that can either transport urate or hexoses alone or exchange urate for glucose or fructose [24]. By acting as a urate transporter, GLUT9 may play an important role in the pathogenesis of metabolic syndrome, obesity, diabetes, hypertension and gout, conditions that have been associated with hyperuricemia [25].

## 2.2 Tissue-specific Regulation of Glucose Transport

### *2.2.1 Intestinal glucose transport*

Enterocytes are the population of intestinal cells responsible for the majority of nutrient absorption and transport. Glucose absorption is the sum of 3 separate processes: transport from the lumen across the brush border membrane (BBM), metabolism within the enterocyte, and finally transport across the basolateral membrane (BLM). The classical model of intestinal glucose transport describes two distinct methods of transport unique to the BBM and BLM of the enterocyte. SGLT-1, thought to be the sole glucose transporter expressed in the BBM, utilizes the energy derived from moving sodium down its concentration gradient to transport luminal glucose across the BBM into the enterocyte. The resulting accumulation of glucose within the enterocyte allows for the facilitative transport of glucose down a concentration gradient across the BLM via GLUT2. For many years, the prevailing theory of intestinal glucose uptake emphasized the role of SGLT1 as the principal mechanism for glucose entry into the enterocyte. However, GLUT2, previously thought to be expressed only in hepatocytes, pancreatic β-cells and the BLM of enterocytes, has also been detected on the apical side of the rat jejunum and shown to be fully functional [26]. A relatively new theory of intestinal glucose transport proposes that GLUT2-mediated transport represents the principle mechanism of glucose absorption on the BBM [27]. Rapid trafficking of GLUT2 to the

apical membrane, controlled by protein kinase C (PKC) $\beta_{II}$ signaling in response to phorbol esters, has also been documented [28]. Based on these results, a new model for intestinal glucose absorption has emerged, whereby GLUT2 translocates to the BBM in response to high luminal glucose concentrations experienced after a meal and, in combination with SGLT-1, contributes to post-meal glucose uptake. It should be noted, however, that these observed mechanisms for glucose uptake in the presence of high luminal glucose concentrations have yet to be confirmed in human intestinal tissue. SGLT-3, while capable of transporting glucose and galactose, is more likely a luminal glucose sensor involved in the release of serotonin by enterochromaffin cells of the mucosa [29]. Interestingly, activity of SGLT-1 and GLUT2 can be upregulated by gut hormones such as glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-2 (GLP-2) [30,31].

GLUT7 is also expressed in the small intestine and colon, although less is known about its relative significance to intestinal glucose transport. In the rat, GLUT7 is localized to the BBM and the highest protein levels are found equally in the jejunum and ileum [32]. Kinetic analysis has revealed an extremely high affinity of GLUT7 for glucose and fructose, with the $K_M$ as low as 200-300 µM. In this light, the kinetics of GLUT7 ensure efficient and complete intestinal glucose absorption, transporting any remaining ileal glucose present 1 to 2 hours after meal consumption where extremely low concentrations would be expected [33]. It is also possible that the very low capacity for glucose and fructose transport exhibited by GLUT7 indicates there may be an alternative physiological substrate for GLUT7 aside from hexoses [32].

Although apical glucose transport in the enterocyte has been extensively studied, less is known about glucose transport at the BLM. The most accepted view of glucose exit involves a GLUT2-dependent mechanism whereby glucose simply travels down its concentration gradient, going from an area of high concentration (inside the enterocyte)

to an area of low concentration (the interstitial space) [34]. However, in patients with Fanconi-Bickel disease where both alleles of GLUT2 are inactive, glucose absorption is not affected [35]. Similarly, GLUT2 knockout mice have a normal glycemic response to an oral glucose load, suggesting that GLUT2 is not required for basolateral transport of glucose [36]. Using an intestinal perfusion model, Stumpel et al. (2001) found that while GLUT2 ablation did not affect glucose absorption, treatment with S4048, a glucose 6-phosphate translocase inhibitor, concentration-dependently decreased glucose absorption [37]. In addition, absorption of [$^{14}$C]3-*O*-methylglucose, a substrate of both SGLT1 and all facilitative glucose transporters which is not phosphorylated by hexokinases, was decreased with GLUT2 ablation. Together, these results support the hypothesis that basolateral glucose transport relies more on translocation of glucose 6-phosphate than on activity of GLUT2. Glucose 6-phosphate must be dephosphorylated before exit through the basolateral membrane by the enzyme glucose 6-phosphatase. Glucose 6-phosphate tranlocase is an important mediator of this process, as this protein transports glucose 6-phosphate from the cytosol to the lumen of the endoplasmic reticulum where the active site of glucose 6-phosphatase is located [38]. In the absence of GLUT2, glucose then exits the enterocyte by vesicular transport mechanisms; in the presence of GLUT2, free glucose most likely re-enters the cytosol and diffuses through GLUT2. However, it is possible that both mechanisms contribute to basolateral glucose transport under normal conditions.

### *2.2.2 Hepatic glucose transport*

Due to its central role in nutrient metabolism, the liver must respond quickly to elevations in blood glucose concentrations. Unlike skeletal muscle and adipose tissue, entry of glucose into the liver is not directly dependent on insulin; however, insulin can indirectly affect hepatic glucose flux by suppressing gluconeogenesis and

glycogenolysis. Glucose transport across the hepatocyte membrane is mediated primarily through GLUT2, a low affinity, high turnover transporter that has a supraphysiological $K_m$ for transport and operates well below saturation [39]. Unlike GLUT4, the facilitative glucose transporter expressed in adipose tissue and skeletal muscle, GLUT2 is constitutively expressed on the plasma membrane and does not require insulin-stimulated pathways to be transported to the plasma membrane. In addition, because of the high number of GLUT2 transporters on the hepatocyte membrane, there is a high maximal activity for glucose transport. Thus, the rate and direction of glucose movement across the hepatocyte membrane are determined by the relative concentrations of intracellular versus extracellular glucose.

Glucose that enters the hepatocyte is immediately phosphorylated by glucokinase, the hepatic isoform of hexokinase, to glucose 6-phosphate. Glucokinase is a key hepatic enzyme, as it catalyzes the formation of the first intermediate in both glycolysis and glycogenesis. Like GLUT2, glucokinase has a high capacity and low affinity for glucose, allowing for maximal phosphorylation at high glucose concentrations. The combined low affinity and high capacity for glucose of both GLUT2 and glucokinase allows the hepatocyte to quickly respond to high blood glucose concentrations that are common in the post-prandial state.

Certain catabolic hormones stimulate hepatic glucose production and therefore indirectly influence the direction of glucose flux in the hepatocyte. For example, binding of glucagon to its G-protein-coupled receptor in hepatocytes increases intracellular cyclic AMP (cAMP) concentrations, releasing the catalytic subunit of protein kinase A (PKA). The catalytic subunit then translocates to the nucleus and influences gene transcription by phosphorylating cAMP response element-binding protein (CREB) at serine 133 [40], thereby allowing recruitment of transcriptional coactivators required for initiation of transcription. Expression of the gluconeogenic enzymes phosphoenolpyruvate

carboxykinase (PEPCK) and glucose 6-phosphatase (Glc6Pase) are dependent on functional CREB activity [41]; thus, in the fasted state, secretion of glucagon from pancreatic α-cells promotes hepatic glucose production by increasing transcription of PEPCK and G6Pase. As a result, intracellular glucose concentrations increase, causing glucose to exit the hepatocyte through GLUT2. Conversely, insulin inhibits hepatic glucose production by suppressing activity of FoxO1, another transcription factor that suppresses transcription of PEPCK and G6Pase [42].

### *2.2.3 Glucose transport in peripheral tissues*

Of the peripheral tissues, skeletal muscle exerts the greatest contribution to postprandial glucose disposal, accounting for approximately 85-90% of post-meal glucose removal from the blood [43,44]. The majority of peripheral glucose uptake into adipose tissue and skeletal muscle is achieved through similar signal transduction pathways and is mediated by the insulin-responsive GLUT4 facilitative glucose transporter. Insulin stimulation causes a 20 to 30-fold increase in the rate of glucose transport across the plasma membrane of adipocytes [45], an effect attributable to the regulation of insulin on GLUT4 translocation to and insertion in the plasma membrane. In unstimulated muscle or adipose cells, 4-10% of GLUT4 is located at the cell surface while greater than 90% is sequestered to intracellular storage vesicles [46]. Therefore, peripheral glucose uptake is dependent on effective insulin-stimulated exocytosis of GLUT4 from intracellular storage vesicles to the plasma membrane. This complex process is initiated when insulin binds to its cognate tyrosine kinase membrane receptor, with consequent activation of the insulin receptor substrate (IRS)/PI3K pathway. In skeletal muscle cells and adipocytes, tyrosine phosphorylation of IRS-1 is required for glucose-stimulated GLUT4 translocation and subsequent glucose uptake [47,48]. Once phosphorylated, IRS-1 binds the regulatory p85 subunit of class I PI3K, which stimulates

the catalytic p110 subunit to produce primarily phosphatidylinositol 3,4,5-triphosphate ($PIP_3$). To a lesser extent, phosphatidylinositol 3,4-diphosphate ($PIP_2$) and phosphatidylinositol 3-phosphate (PIP) are produced by class II and class III PI3Ks, respectively. While production of $PIP_2$ does not appear to play a role in GLUT4 trafficking, production of both $PIP_3$ and PIP is required for effective mobilization and fusion of GLUT4-containing vesicles with the plasma membrane [49].

Three major signaling arms, each contributing to GLUT4 mobilization and membrane insertion, lie downstream of class I PI3K: Akt, atypical PKC (aPKC) and Rac. Perhaps the most well-characterized of these is Akt, whose role in GLUT4 mobilization is well-established and has been substantiated by both loss and gain of function studies in adipocytes as well as muscle cells. Of the Akt isoforms, Akt2 appears to govern GLUT4 translocation [50-55] by activating several substrates, including Akt substrate of 160 kDa (AS160) [56-59] and TBC1D1, two Rab-GTPase activating proteins (Rab-GAPs). Rab GTPases regulate many steps of membrane trafficking, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion [60].

Although the exact mechanisms remain unknown, both aPKC (specifically the ζ and λ isoforms) and Rac are involved in GLUT4 vesicle transport via effects on cytoskeletal remodeling, specifically by influencing microtubule and microfilament dynamics. Microtubules and microfilaments are necessary structural elements for GLUT4 insertion into the plasma membrane: microtubules assist trafficking of intracellular GLUT4 storage vesicles to the cell periphery as well as docking to the plasma membrane, [46,61-63], while filamentous actin regulates GLUT4 vesicle tethering, docking and fusion. Stimulating adipocytes or skeletal muscle cells with insulin results in a remodeling of cortical actin filaments that reside just under the plasma membrane, causing a phenomenon known as “membrane ruffling” [64]. Membrane ruffling is essential for effective tethering, docking and insertion of the GLUT4 vesicles to

the plasma membrane, as disruption of cortical actin with agents such as latrunculin B or *Clostridium difficile* toxin B dose-dependently reduce GLUT4 exocytosis as well as rates of glucose uptake [65]. On the other hand, the PI3K substrate $PIP_2$ can stimulate actin polymerization, thereby promoting optimal movement and/or fusion of GLUT4-containing vesicles to the plasma membrane [66].

## 2.3 Diabetes Mellitus

Diabetes mellitus (DM) is a chronic disease characterized by deficits in the physiological actions of the hormone insulin, caused by either insulin deficiency or cellular insulin resistance. DM is classified based on the nature of insulin insufficiency. Type 1 DM (T1DM) is characterized by autoimmune destruction of pancreatic β-cells in genetically predisposed individuals, leading to reduced insulin production and secretion. Effective management of T1DM can be achieved with exogenous insulin doses combined with careful dietary management. Ninety percent of all cases of diabetes mellitus are type 2 DM (T2DM). Although the exact mechanisms are complex, multifaceted and still not completely elucidated, genetic predisposition, excess caloric intake and physical inactivity contribute to the development of cellular insulin resistance and disruption of normal insulin receptor signaling pathways. Compromised insulin receptor signaling impairs the ability of adipocytes and skeletal muscle cells to clear glucose from the bloodstream, contributing to hyperglycemia. In addition, the loss of the suppressive effect of insulin on hepatic gluconeogenesis further exacerbates hyperglycemia. Initially, pancreatic β-cells will compensate for excess blood glucose by increasing production and secretion of insulin; however, compensatory hyperinsulinemia is a transient phenomenon and eventually insulin secretion is reduced as β-cell function declines.

Unmanaged hyperglycemia leads to several complications including cardiovascular disease (CVD), nephropathy, neuropathy and retinopathy. According to the World Health Organization there are currently 346 million people worldwide who have DM [67]. DM not only places an immense burden on the health care system by draining monetary and human resources, but it also significantly compromises the quality of life for patients and their families and reduces life expectancy. Among adults aged 20 years or older, the mortality rate for those with DM is twice as high as those without [68]. Reduced life expectancy is not confined solely to elderly patients: men and women having DM between the ages of 20 and 39 years live an average of 9 and 8 years less, respectively, than those without DM [68]. Additionally, increased obesity in children and youth has resulted in an alarming rise in the incidence of T2DM among those below the age of 18 [67].

## 2.4 Glucose Metabolism in T2DM

### *2.4.1. Intestinal glucose transporters in diabetes and insulin-resistant states*

The first observed pathological effects of diabetes on the intestine were documented in 1942, when it was discovered that experimentally-induced T1DM in rats led to higher rates of intestinal sugar absorption [69]. Since then, a number of studies have confirmed this finding in both T1DM [70-74] and T2DM [75]. Initially, it was believed that increased intestinal absorption occurred secondary to intestinal hyperplasia that occurs in diabetes. The perceived state of starvation elicited by reduced cellular glucose in peripheral tissues causes an increase in mucosal mass, a compensatory attempt by the intestine to absorb more glucose. However, increases in glucose transport appear well before mucosal changes can be detected [73,76], and increased rates of glucose uptake persist after correction for mucosal dry weight [77]. Therefore,

other mechanisms must contribute to the increase in glucose transport rates induced by diabetes.

Streptozotocin-induced T1DM in male Sprague Dawley rats is associated with higher levels of GLUT2, GLUT5 and SGLT-1 mRNA and protein in both the jejunum and ileum [78]. Insulin treatment of streptozotocin-induced T1DM reduces hyperglycemia and restores glucose transporter expression to near normal levels; however, it is unknown if this restoration is due to direct effects of insulin or reduction in glycemia. Up-regulation of intestinal GLUT5 and GLUT2 in models for T1DM has been observed in other studies [26], solidifying the evidence for up-regulation of transporter expression as a contributing factor to the enhanced glucose transport rates that have been observed in this disease. In one of the few studies that has examined the effects of T2DM, no differences in expression of SGLT-1, GLUT2 or GLUT5 were found in mucosal scrapings from lean Zucker and Zucker Diabetic Fatty (ZDF) rats [79]. Research in humans with T2DM has found that higher expression of SGLT-1 and GLUT5, but not GLUT2, accompanies elevated rates of glucose uptake in isolated BBM vesicles prepared from duodenal biopsies [80]; however, GLUT2 mRNA was elevated in the total duodenum, suggesting that T2DM in humans is also associated with higher expression of GLUT2 in the BLM. Therefore, while it is accepted that experimentally-induced T1DM is associated with higher expression of intestinal active and facilitative glucose transporters, more research is required to definitively determine the effect of T2DM and insulin resistance on transporter expression.

Activity of intestinal glucose transporters is also affected in DM. Madsen et al. (1996) demonstrated that the increased SGLT-mediated transport observed in T1DM is associated with higher maximal glucose transport rates in isolated jejunal and ileal BBM vesicles [81]. This effect could not be attributed to T1DM-induced hyperphagia and the consequent increased delivery of nutrients to enterocytes, as rats with T1DM fasted for

12 hours also displayed elevated rates of intestinal glucose transport. In this study, insulin injection resulted in amelioration of elevated glucose transport and maximal transport activity 12 hours post injection, with a full reduction to control levels after 48 hours. Reversal of impaired intestinal glucose transport elicited by experimentally-induced diabetes using insulin treatment has been previously documented [82,83], although this does not identify insulin or the reduction in hyperglycemia secondary to insulin therapy as the primary cause of this reversal. However, normalization of jejunal and ileal glucose transport through insulin therapy observed by Madsen et al. (1996) occurred prior to the reduction in hyperglycemia, a phenomenon which has been reported previously [84] and suggests that the intestine may be responding directly to insulin. Indeed, insulin receptors are present along the entire intestinal tract [85], making the direct regulation of intestinal glucose transporters by insulin plausible.

Using polarized Caco2-Tc7 cells, a cell line often used to study regulation of intestinal transport *in vitro*, Tobin et al. (2008) provided additional evidence for the independent regulation of intestinal GLUT2 by insulin [86]. One hour of insulin treatment in these cells resulted in an 80% reduction in BBM GLUT2. Similarly, insulin treatment was able to reduce BLM GLUT2 protein, even in the presence of 25 mM glucose. This insulin-stimulated internalization of GLUT2 from both the BBM and BLM resulted in a 30% reduction in 3-O-methylglucose transport. Similar results were found with insulin-sensitive mice: under hyperinsulinemic euglycemic clamp conditions, insulin promoted internalization of GLUT2 from the BBM. Furthermore, high fat- or high fructose-induced insulin resistance in mice led to higher GLUT2 levels in the basal state and impaired insulin-stimulated GLUT2 internalization. Therefore, insulin resistance could lead to a loss of insulin regulation of GLUT2 membrane trafficking, leading to a permanent localization of GLUT2 in the enterocyte BBM and a significant contribution to increased intestinal glucose transport in T2DM.

### *2.4.2 Effects of insulin resistance and diabetes on glucose transporter activity in peripheral tissues*

2.4.2.1 Adipose tissue/adipocytes

In adipocyte cell lines, as well as primary adipocytes of human and non-human origin, insulin resistance and diabetes reduce rates of both basal and insulin-stimulated glucose uptake [87-89]. Impaired glucose uptake in obesity and DM may arise from a number of possible scenarios, acting alone or in concert: reduced expression of glucose transporters, attenuated translocation of transporters to the plasma membrane or diminished activity of the transporters themselves. Indeed, T1DM in rodents has been associated with reduced levels of adipose tissue GLUT4 mRNA and protein, an effect that is reversed within 48 hours of initiating insulin therapy [90,91].

To characterize the defect in glucose transport that contributes to reduced rates of glucose uptake observed in humans, Garvey et al. [88] isolated adipocytes from lean and obese volunteers, as well as those with T2DM. Using the cytochalasin B binding assay, a measure of the chemical affinity of all facilitative glucose transporters for cytochalasin B, it was found that adipocytes from only the volunteers with T2DM had lower numbers of glucose transporters in the plasma membrane during the basal state; however, upon insulin stimulation, adipocytes from both the obese and T2DM groups had reduced numbers of plasma membrane transporters. These data suggest that both obesity and T2DM reduce glucose transporter expression. To assess the functionality of these transporters, plasma membrane transporter concentration was expressed relative to the transport rate per cell surface area. Despite no difference in quantity of glucose transporters in the plasma membrane of lean and obese, the basal glucose transport rate of adipocytes was 76% lower in obese individuals. In T2DM, the transport rate corrected for cell surface area was reduced to a greater extent than transporter number.

Therefore, in both the obese and T2DM groups, reduced rates of basal glucose transport could not be entirely accounted for by the reduction in transporter number. The same pattern of results was found for insulin-stimulated glucose transport: in both the obese and T2DM groups, rates of transport were reduced to a greater extent than the reduction in transporter number, suggesting that in addition to reduced quantity, transporter activity is also impaired in these states [87]. Subsequent studies determined a significant reduction in GLUT4 mRNA and protein in adipocytes from obese individuals and T2DM contributed to reduced glucose uptake [88].

Follow-up studies on the mechanism for reduced rates of glucose uptake in adipocytes have relied heavily on the convenience of using cell lines to manipulate various experimental conditions. Differentiated 3T3-L1 adipocytes respond to insulin stimulation by increasing the rate of glucose transport 10-20 fold above basal levels, making them an ideal model to study the mechanics of insulin-stimulated GLUT4 translocation [92,93]. Undoubtedly, a fundamental defect leading to failed glucose transporter translocation is the absence of signal emanating from the insulin receptor, either from lack of insulin (T1DM) or dysfunction of the signaling mediators (T2DM). Attenuated activity of insulin signaling intermediates, while fundamental to the failure of GLUT4 translocation, are not the primary focus of this review, although this has been the subject of many excellent papers [94-96].

Chronic exposure of 3T3-L1 adipocytes to 100 nM insulin, to mimic hyperinsulinemia, leads to reduced cell surface levels of GLUT4 and reduces the rate of insulin-stimulated glucose transport [89], presumably due to the markedly attenuated expression and/or activity of the insulin receptor, IRS-1, PI3K, Akt and GLUT4 [97,98]. However, pharmacological doses of insulin may not accurately reflect physiological concentrations of the circulating hormone, especially in the early stages of insulin resistance that precede compensatory hyperinsulinemia. Studies employing

physiologically relevant doses of insulin (around 5 nM) in 3T3-L1 cells have demonstrated an attenuation of insulin-stimulated GLUT4 translocation and reduced glucose uptake, despite no changes in expression or activity of early insulin signaling intermediates or expression of GLUT4 [99,100]. Chronic exposure to insulin does, however, lead to a profound loss of cortical filamentous actin and $PIP_2$ in the plasma membrane, leading to impaired membrane ruffling and insertion of GLUT4 vesicles into the plasma membrane [99]. Interestingly, restoration of $PIP_2$ corrects all insulin resistance-associated defects in GLUT4 insertion. Furthermore, chronic treatment with low levels of insulin leads to higher levels of GLUT4 in the plasma membrane in the basal state without a parallel increase in basal rates of glucose transport, suggesting compromised function of GLUT4 in insulin-resistant adipocytes [99].

2.4.2.2 Skeletal muscle

As with adipocytes, rates of glucose transport in insulin resistant or diabetic skeletal muscle are reduced, regardless of the nature of insulin signaling deficits [101-103]. However, in contrast to adipose tissue and adipocytes, the contribution of reduced transporter expression to impaired glucose uptake is controversial. Reduced levels of GLUT4 (but not GLUT1) mRNA in skeletal muscle of diabetic rats have been reported by some groups [101,104], but not others [105,106]. In humans, GLUT4 and GLUT1 mRNA levels do not appear to be affected by obesity, T1DM or T2DM; furthermore, there is no correlation between GLUT4 protein or mRNA and degree of glycemic control, fasting plasma glucose, fasting plasma insulin, or duration of diabetes [107]. This discrepancy could result from the use of different muscle groups for analysis in animal versus human studies. Traditionally, animal studies have analyzed hindlimb muscles, composed primarily of type I and type IIa fibres. Studies using muscle from humans, however, analyze specimens easily obtained by biopsy, such as the vastus lateralis or

rectus abdominus muscles, which are composed primarily of type IIb fibres. Both the capacity for insulin-stimulated glucose transport and tissue content of GLUT4 is dependent on the type of muscle group and the muscle fibre type of which it is composed [108,109], presenting a potential confounding variable when attempting to compare results between humans and animals. To address this issue, Hardin et al. (1993) isolated soleus, gastrocnemius, vastus laterails, rectus abdominus and cardiac muscle from Sprague-Dawley rats with streptozotocin-induced T1DM. Induction of T1DM resulted in an approximate 50% reduction in GLUT4 protein levels in the soleus, gastrocnemius and cardiac muscles, but GLUT4 protein levels in rectus abdominus and vastus lateralis muscles were reduced by only 18%. After insulin treatment, GLUT4 protein levels in cardiac, soleus and gastrocnemius muscles were higher than untreated controls, but were not elevated in the vastus lateralis and rectus abdominus muscles [110], supporting the idea that alterations in GLUT4 expression and regulation by insulin are strongly influenced by muscle fibre type.

In addition to altered expression of transporters, insulin resistance in skeletal muscle can affect GLUT4 translocation. Soleus and gastrocnemius muscle from obese, insulin-resistant *fa/fa* Zucker rats is characterized by reduced glucose uptake, a lower maximal transport rate for facilitative glucose transport and a failure of insulin to increase plasma membrane GLUT4 content [102]. Molecular studies, the majority of which have used differentiated L6 skeletal muscle cells, have yielded insight into the molecular mechanisms governing diminished GLUT4 translocation in insulin-resistant states. Much like 3T3-L1 adipocytes, chronic treatment of differentiated L6 myotubes with insulin for 12 to 24 hours can produce an *in vitro* model of insulin-resistant skeletal muscle. Huang et al. (2002) demonstrated that incubating L6 myotubes in high glucose (25 mM) and high insulin (100 nM) for 24 hours leads to increased basal glucose uptake, but reduces insulin-stimulated 2-deoxyglucose uptake [111]. Using L6 cells stably

expressing GLUT4 that carries an exofacial myc-epitope, these researchers found that cells pre-treated with high glucose/high insulin experienced a 45% reduction in the cell surface levels of GLUT4myc after acute insulin stimulation. The high glucose/high insulin pretreatment of these cells was associated with reduced protein levels and phosphorylation of the insulin receptor, IRS-1, PI3K and Akt, suggesting that attenuation of insulin signaling activity is responsible for the reduction in GLUT4 translocation to the plasma membrane and subsequent glucose uptake. Interestingly, the high glucose/high insulin treatment did not change surface GLUT4myc levels under basal conditions despite increasing 2-deoxyglucose uptake by 40%. This increase in basal glucose uptake could not be fully accounted for by increased cellular levels of GLUT1. The researchers did observe upregulation of protein levels, phosphorylation and activity of p38 mitogen activated protein kinase (MAPK), suggesting a role for this signaling pathway in the upregulation of basal glucose uptake induced by a combination of high glucose and high insulin.

Chronic exposure to supraphysiological concentrations (100 nM) of insulin can down-regulate expression and activity of the insulin receptor, IRS-1, PI3K and Akt [98,111,112,112]; this loss of insulin signaling may be responsible for decreased translocation of GLUT4 to the plasma membrane for subsequent uptake of glucose into the cell. However, chronic exposure of exofacial myc-epitope GLUT4-expressing L6 cells to lower concentrations (5 nM) of insulin also resulted in significantly reduced insulin-stimulated glucose uptake and GLUT4myc staining, in the absence of impaired Akt phosphorylation [113]. Furthermore, the ability of Akt to phosphorylate AS160 was not different between control cells and those treated chronically with insulin. Thus, in this study, attenuation of early and distal insulin signaling is not responsible for impaired glucose uptake observed with exposure of skeletal muscle cells to physiologically-relevant concentrations of insulin. Similar to what occurs in adipocytes, chronic exposure

of L6 myotubes to low concentrations of insulin has been shown to reduce cortical actin around the cell periphery and produce a selective loss of filamentous actin. Furthermore, attenuated insulin-stimulated glucose uptake, GLUT4 translocation and cortical actin expression in L6 myotubes were observed at both low and high concentrations of glucose, supporting a hypothesis that insulin-induced insulin resistance acts independently of glucose load and thus represents a cellular defect that may significantly contribute to the worsening and progression of insulin resistance [113]. It is interesting to note that the findings from this study *in vitro* also translated to the situation *in vivo*, as epitrochlearis muscle isolated from insulin-resistant *fa/fa* Zucker rats had reduced levels of cortical filamentous actin compared to lean controls.

## 2.5 Incretin Hormones in T2DM

The gastrointestinal tract contains endocrine cells that secrete hormones necessary for the regulation of digestion. Specialized populations of enteroendocrine cells secrete the hormones responsible for the incretin effect, or the phenomenon whereby orally administered glucose elicits a larger insulin response compared to an equivalent amount of intravenous glucose [114]. This effect has been attributed to the actions of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), products of the proglucagon and proGIP genes, respectively [114]. By interacting with G-protein coupled receptors on pancreatic β-cells, incretins stimulate insulin secretion via PKA signaling and altering membrane polarization and $Ca^{2+}$ flux [115,116]. Deletion of the GIP and GLP-1 receptors in pancreatic β-cells or use of receptor antagonists leads to glucose intolerance, demonstrating a fundamental role for incretin hormones in normal glucose metabolism [117]. Several studies have demonstrated that people with T2DM have a blunted incretin response [118,119], primarily due to a significant (upwards of 50%) reduction in post-prandial GLP-1 secretion [120]. Unlike

GIP, the insulinotropic effect of both endogenous and exogenous GLP-1 is retained in T2DM [121,122].

## 2.6 Gastrointestinal Satiety Hormones in T2DM

### *2.6.1 Pancreatic polypeptide*

Pancreatic polypeptide (PP) is a 36 amino acid peptide belonging to the PP-fold family of proteins. Release of PP from F cells of the pancreas [123] is proportional to caloric content of a meal, but is also stimulated by other gastrointestinal hormones such as cholecystokinin, ghrelin, motilin, and secretin [124]. Physiological effects of this hormone include delayed gastric emptying, as well as inhibition of gallbladder motility and pancreatic secretions [125]. Additionally, PP has demonstrated anorexigenic effects in both mice and humans [126,127] by activating Y4 receptors in appetite-regulating centres of the brainstem and the hypothalamus [128]. Little information exists regarding the relationship between PP and diabetes, although some associations have been noted. Higher numbers of PP-positive cells have been reported in older ZDF rats compared to their lean counterparts [129]. In humans, elevated first and second phase PP secretion has been documented in Pima Indians, a population with high incidences of obesity, insulin resistance and T2DM [130]. A relationship between T2DM and PP is also highlighted by studies showing improvements in insulin sensitivity secondary to weight loss are accompanied by reduced PP concentrations [131]. However, these studies do not identify changes in PP as the cause or consequence of insulin resistance and T2DM.

### *2.6.2 Peptide YY*

Peptide YY (PYY) is synthesized as a 36 amino acid polypeptide, but is subject to cleavage by dipeptidyl peptidase IV to produce $PYY_{3-36}$, the main circulating form in

both the fasted and fed states [132]. Peptide YY is secreted from L cells of the distal gastrointestinal tract [133]. Circulating PYY increases in response to several factors, including caloric load, nutrient composition and food consistency [134-136], with maximal concentrations achieved 1 to 2 hours post-meal [137]. Although several studies support a relationship between PYY and insulin resistance and/or T2DM, the nature of this relationship is not completely clear. Studies using animal models for insulin resistance and T2DM strongly suggest a beneficial effect of PYY on insulin resistance and its associated metabolic derangements. For example, several hours of intravenous PYY administration improved insulin sensitivity in diet-induced insulin-resistant mice [138], while four weeks of subcutaneous infusion of $PYY_{3-36}$ in ZDF rats as well as mice and rats with diet-induced insulin resistance improved glycated hemoglobin (HbA1c) and glucose tolerance and reduced fasting serum insulin [139,140]. In humans, however, elevated HbA1c, fasting plasma glucose and impaired glucose tolerance are present despite elevated plasma PYY concentrations, suggesting the presence of PYY resistance in these states. [141]. Although fasting levels of PP are elevated, post-prandial secretion of PYY appears to be attenuated in T2DM [142]. Even normal weight, insulin-sensitive individuals with a strong family history of T2DM experience diminished post-prandial secretion of PYY in response to a high carbohydrate meal [143]. Modest associations between a common Arg72Thr variant in the human PYY gene and T2DM have been discovered [144].

### *2.6.3 Ghrelin*

Ghrelin is a 28 amino acid peptide secreted from X/A cells of the gastric fundus [145]. Addition of octanoyl acid to serine 3 is essential for ghrelin activity [146]. Ghrelin is a potent stimulator of appetite [147] by activating neuropeptide Y and Agouti-related protein-expressing neurons in the arcuate nucleus of the hypothalamus [148].

Intravenous infusion of ghrelin increases appetite in both lean and obese individuals [149,150]. Levels of ghrelin increase during fasting and decline rapidly after eating, suggesting a role in meal initiation [151]. Circulating ghrelin levels are low in obese people and high in those with anorexia nervosa, but normalize once body mass starts to approach normal levels [152]. However, post-prandial ghrelin levels do not rapidly decline as in healthy individuals, which may explain the reduced satiety after food intake that has been observed in obesity [153]. Fasting plasma concentrations of ghrelin are significantly lower in individuals with T2DM and insulin-resistant obese individuals, even after adjustment for age, sex and body mass index (BMI) [154,155]. A recent study that aimed to clarify the relationships between total, acylated and de-acylated ghrelin molecules specifically with insulin resistance showed that these molecules might be involved in glucose and insulin metabolism in different ways. Total and de-acylated ghrelin were negatively associated with insulin resistance, while there was a positive correlation between acylated ghrelin and homeostasis model of assessment of insulin resistance (HOMA-IR) [156].

## 2.7 Management of T2DM

Diabetes management focuses on improving glycemic control due to its direct relationship with microvascular and macrovascular complications, especially CVD. Glycated hemoglobin (HbA1c) is the clinical marker used to assess long-term blood glucose control, as chronic elevations in both fasting and post-prandial blood glucose concentrations lead to higher rates of non-enzymatic glycation of hemoglobin [157,158]. In both T1DM and T2DM, the goal of therapy is to reduce HbA1c to at least 7.0% [159].

Weight loss and exercise are important non-pharmacological approaches to improving glycemic control. Caloric restriction and exercise have additive effects on glycemic control; as such, individuals with T2DM are encouraged to engage in at least

150 minutes of moderate-intensity aerobic exercise per week [160]. However, if weight loss through lifestyle modification does not reduce HbA1c, or if an individual presents with an initial HbA1c greater than 9.0%, pharmacological therapy is initiated. Metformin is usually the first pharmacological agent prescribed, due to its effectiveness in lowering blood glucose, minimal side effects and potential weight-reducing effects. This drug acts by stimulating AMP-activated protein kinase (AMPK) with consequent suppression of PEPCK and Glc6-Pase gene expression [161], thereby suppressing hepatic glucose production. If ineffective as a monotherapy, metformin may be prescribed in combination with one of several other oral anti-hyperglycemic agents that work through different mechanisms. Alpha-glucosidase inhibitors reduce post prandial glucose excursions by inhibiting intestinal breakdown of starch. Sulphonylureas and meglitinides, known collectively as insulin secretagogues, stimulate insulin secretion through closure of specific potassium channels on pancreatic β-cells [162]. Thiazolidinediones activate peroxisome proliferator-activated receptor γ and enhance insulin sensitivity and glucose uptake in peripheral tissues [163].

Newer classes of oral antihyperglycemic medications include incretin mimetics and inhibitors of SGLT-2. Incretin mimetics, structural analogues of GLP-1, promote weight loss in addition to improving HbA1c [164]. DPP-IV inhibitors, which block the degradation of endogenous GLP-1 thereby modestly increasing circulating GLP-1 levels, are also in use [163]. SGLT-2 inhibitors selectively block glucose reabsorption by the kidneys, thereby increasing urinary glucose excretion and thus reducing blood glucose concentrations. Phase III clinical trials on dapagliflozin support beneficial dose-dependent effects of SGLT-2 inhibitors on fasting blood glucose, glucose tolerance and HbA1c as well as weight loss [165].

## 2.8 Buckwheat

### *2.8.1 Nutritional properties*

Buckwheat refers to plants belonging to the Polygonaceae family. Common buckwheat (*Fagopyrum esculentum* Moench) is a broad-leafed herbaceous annual plant, thought to have originated in southern China. Another buckwheat species, tartary buckwheat (*Fagopyrum tataricum* Gaertn) or "bitter buckwheat" is also used as a crop, but it is much less common. Contrary to its name, buckwheat is not related to wheat; however, it is often classified and treated as a cereal due to the structural and chemical similarities of its seeds [166].

For many years, buckwheat has been grown as a food crop and is part of the diet of people worldwide. Within Canada, Manitoba produces 70% of Canada's buckwheat crop [167]. Varieties of buckwheat grown in Manitoba include AC Springfield, AC Manisoba, Mancan, Manor, AC Manisoba, Koma and Koto. Koto buckwheat is of special interest to food developers due to increased starch content and soft starch characteristics [166]. Buckwheat seeds are triangular in shape and are encased by a hull, also known as the pericarp. Once the hull is removed, the seed is referred to as a groat, which is similar to a cereal kernel in regards to chemical composition and structure. The first layer of the groat is a one-cell thick testa layer (seed coat); under the testa is a one-cell aleurone layer, which surrounds a starchy endosperm. The inner portion of the groat consists of a spermaderm and an endosperm. A large embryo and two cotyledons are contained in the center of the endosperm [168].

Buckwheat groats contain less than 5% fat [169], which is composed of 16-20% saturated fatty acids, 30-45% oleic acid and 31-41% linoleic acid. Palmitic, oleic, linoleic and linolenic acids account for about 95% of buckwheat total fatty acids. Starch is the major carbohydrate in buckwheat, and its amount in Canadian buckwheat varieties may vary from 67-75%. The dietary fibre content can range from 5-11%, and includes

cellulose, lignins and non-starch polysaccharides [170]. Buckwheat also contains a small proportion of resistant starch [171]. Compared to cereals, buckwheat has a well-balanced amino acid profile and is rich in lysine, which is usually considered the first limiting amino acid in wheat and barley. The protein of buckwheat flour has an amino acid score of 100, one of the highest amino acid scores among plant sources [172]. Despite the balanced amino acid composition, digestibility of buckwheat protein in humans and animals is relatively low [173,174] due to the presence of anti-nutritional factors such as protease inhibitors and tannins [175].

Buckwheat is a source of many minerals, including potassium, phosphorus, magnesium, calcium, iron, manganese, boron and copper. Compared to cereals such as rice, wheat, flour or corn, buckwheat contains higher levels of zinc, copper and manganese [176,177]. Thiamin, riboflavin and niacin are also found in buckwheat flour at relatively high levels [168]. In addition to traditional nutrients, buckwheat is a source of many phytochemicals, particularly flavonoids. Rutin, a flavonol glycoside, is the main flavonoid found in buckwheat [178]. Other flavonoids that have been isolated from buckwheat include orientin, vitexin, quercetin, isovitexin, quercetrin and isorientin. Flavonoid content and composition in buckwheat seeds is affected by a number of variables including growing phase and growing conditions. Species also influences flavonoid content: tartary buckwheat contains approximately 40 mg/g total flavonoids, while common buckwheat contains as little as 10 mg/g [166].

### *2.8.1 Buckwheat in glycemic management*

Epidemiological evidence from studies conducted in rural Chinese communities has demonstrated a potential role for buckwheat in the management of hyperglycemia. In a study of the Kulun and Kezhuohou communities, it was shown that the Kulun community, who consumed 63% of their total grain intake from buckwheat, had

significantly lower age- and BMI-adjusted fasting glucose concentrations compared to the Kezhuohou community, whose major grain source was corn [179]. In addition, the adjusted prevalence rate of hyperglycemia in Kulun participants was 1.91% [(95% confidence interval (CI): 0.67%, 3.15%], which was significantly lower than the rate of 7.33% (95% CI: 5.02%, 9.64%) in Kezhuohou participants. Although not statistically significant, the prevalence of T2DM in Kulun participants was 0.85% (95% CI: 0.02%, 1.68%) compared to 1.43% in the Kezhuohou participants.

Intervention studies also support a role for buckwheat in glycemic management. In a study of 78 individuals with T2DM who consumed 30% of their grain intake as tartary buckwheat flour for up to 3 months, 58% experienced a decline in fasting blood glucose concentrations [180]. Reductions in blood glucose concentrations could be observed as soon as one month after consuming buckwheat. Unfortunately, this study did not include a group of participants not consuming buckwheat or a healthy group of participants consuming buckwheat. Similarly, a separate study demonstrated that participants with T2DM who consumed biscuits prepared with tartary buckwheat flour experienced a 3.71 mmmol/L (36%) reduction in blood glucose concentrations. In a separate group given buckwheat biscuits in combination with glybenzcyclamide, an α-glucosidase inhibitor, blood glucose concentrations were reduced by 4.34 mmol/L (37%) [181].

A small number of studies have examined potential mechanisms contributing to reduced glycemia observed with buckwheat consumption. Several flavonols, including myricetin, quercetin, kaempferol and fisetin, have shown significant inhibitory actions on α-glucosidase and α-amylase [182], two enzymes involved in the digestion of starch in the small intestine. Similarly, a buckwheat bran extract made from common buckwheat has been shown to significantly inhibit sucrase activity *in vitro* [183]. Furthermore, administration of the same buckwheat bran extract, or an extract made from whole

buckwheat, to Balb/c mice 30 minutes prior to an oral sucrose challenge led to significantly lower blood glucose concentrations 15, 30 and 60 minutes after the sucrose challenge [183]. Although the inhibition of sucrase was hypothesized to stem from the inhibitory actions of rutin, exposure of mice to rutin 30 minutes before a sucrose challenge did not significantly affect post-prandial glucose concentrations, suggesting that another compound in the buckwheat extract was responsible for inhibiting sucrase activity [183]. Attenuated post-prandial glucose concentrations were not observed when mice were challenged with maltose or glucose, indicating that the inhibitory effect was specific for sucrase.

Buckwheat is a source of D-*chiro*-inositol (D-CI) and *myo*-inositol (MI), two members of the inositol phosphoglycan family [184]. In both T1DM and T2DM, urinary excretion of D-CI is greatly elevated, and is positively associated with fasting plasma glucose, glucosuria and HbA1c, suggesting altered metabolism of D-CI in DM [185]. Inositiol phosphoglycans have been shown to possess anti-hyperglycemic effects. Chemically synthesized D-CI, when administered intragastrically, reduced plasma glucose concentrations by 30-50% in normal rats [186]. Although not investigated in that study, the reduction in plasma glucose by D-CI may have been due to stimulation of insulin-responsive glucose uptake pathways, as D-CI and MI are known to possess insulin-mimetic properties [187]. When administered at a dose based on D-CI content, a concentrate produced from common buckwheat of the Koto variety (20 mg of D-CI per kg body weight) reduced blood glucose concentrations by 12% after 90 minutes and by 19% after 120 minutes in a rat model for T1DM [188]. In addition, oral glucose tolerance was improved, although this did not reach statistical significance when compared to type 1 diabetic rats given a sucrose solution. The lower efficacy of the buckwheat concentrate to reduce blood glucose concentrations when administered 60 minutes before an oral glucose load may relate to the mechanism of action of buckwheat:

buckwheat may exert acute effects on glucose uptake, and perhaps administering the buckwheat concentrate closer to the glucose dose would have yielded significant results. The mechanism for reduced blood glucose induced by the buckwheat concentrate could not be determined from this study; however, it was hypothesized that reduction in glycemia was due to D-CI and MI present in the buckwheat. Similarly, an extract produced from D-CI-enriched tartary buckwheat dose-dependently reduced fasting blood glucose concentrations in KK-Ay mice, a model for T2DM, after 2 weeks of oral administration. Furthermore, after 5 weeks of treatment, KK-Ay mice had significantly lower circulating C-peptide and glucagon, and improved insulin immunoreactivity in pancreas [189].

To assess the insulin-mimetic properties of buckwheat, Kawa et al. [190] examined the effects of a Koto buckwheat concentrate containing D-CI and MI, as well as isolated D-CI and MI, on activation of key intermediates involved in insulin signaling. As expected, treatment of H4IIE cells (a rat hepatoma cell line) with insulin resulted in increased phosphorylation of mediators of the insulin signaling pathway, such as the insulin receptor, insulin receptor substrate-1, Akt, Src, STAT3 and glycogen synthase kinase 3. However, treatment with D-CI, MI or the buckwheat concentrate did not result in phosphorylation of these proteins. Interestingly, phosphorylation of p42/44 extracellular regulated kinase (p42/44 ERK) was observed with insulin and buckwheat stimulation, but not with D-CI or MI treatments. These results suggest that attenuation of hyperglycemia by the buckwheat concentrate is not mediated by D-CI and/or MI, but by some novel bioactive working through an undetermined mechanism. Interestingly, it was also observed that treating H4IIE cells with the buckwheat concentrate before exposure to glucose significantly inhibited glucose uptake, even in the presence of insulin.

## 2.9 Literature Cited

[1] Walmsley, A.R., Barrett, M.P., Bringaud, F., and Gould, G.W. (1998). Sugar transporters from bacteria, parasites and mammals: structure-activity relationships. *Trends Biochem. Sci.* 23, 476-81.

[2] Hediger, M.A., Coady, M.J., Ikeda, T.S., and Wright, E.M. (1987). Expression cloning and cDNA sequencing of the Na+/glucose co-transporter. *Nature* 330, 379-81.

[3] Hediger, M.A., Turk, E., and Wright, E.M. (1989). Homology of the human intestinal Na+/glucose and Escherichia coli Na+/proline cotransporters. *Proc. Natl. Acad. Sci. U. S. A.* 86, 5748-52.

[4] Coady, M.J., Pajor, A.M., and Wright, E.M. (1990). Sequence homologies among intestinal and renal Na+/glucose cotransporters. *Am. J. Physiol.* 259, C605-10.

[5] Wells, R.G., Pajor, A.M., Kanai, Y., Turk, E., Wright, E.M., and Hediger, M.A. (1992). Cloning of a human kidney cDNA with similarity to the sodium-glucose cotransporter. *Am. J. Physiol.* 263, F459-65.

[6] You, G., Lee, W.S., Barros, E.J., Kanai, Y., Huo, T.L., Khawaja, S., Wells, R.G., Nigam, S.K., and Hediger, M.A. (1995). Molecular characteristics of Na(+)-coupled glucose transporters in adult and embryonic rat kidney. *J. Biol. Chem.* 270, 29365-71.

[7] Kong, C.T., Yet, S.F., and Lever, J.E. (1993). Cloning and expression of a mammalian Na+/amino acid cotransporter with sequence similarity to Na+/glucose cotransporters. *J. Biol. Chem.* 268, 1509-12.

[8] Dunham, I., Shimizu, N., Roe, B.A., Chissoe, S., Hunt, A.R., Collins, J.E., Bruskiewich, R., Beare, D.M., Clamp, M., Smink, L.J., Ainscough, R., Almeida, J.P.,

Babbage, A., Bagguley, C., Bailey, J., Barlow, K., Bates, K.N., Beasley, O., Bird, C.P., Blakey, S., Bridgeman, A.M., Buck, D., Burgess, J., Burrill, W.D., and O'Brien, K.P. (1999). The DNA sequence of human chromosome 22. *Nature* 402, 489-95.

[9] Diez-Sampedro, A., Eskandari, S., Wright, E.M., and Hirayama, B.A. (2001). Na+-to-sugar stoichiometry of SGLT3. *Am. J. Physiol. Renal Physiol.* 280, F278-82.

[10] Wright, E.M. (2001). Renal Na(+)-glucose cotransporters. *Am. J. Physiol. Renal Physiol.* 280, F10-8.

[11] Birnir, B., Lee, H.S., Hediger, M.A., and Wright, E.M. (1990). Expression and characterization of the intestinal Na+/glucose cotransporter in COS-7 cells. *Biochim. Biophys. Acta* 1048, 100-4.

[12] Mackenzie, B., Loo, D.D., and Wright, E.M. (1998). Relationships between Na+/glucose cotransporter (SGLT1) currents and fluxes. *J. Membr. Biol.* 162, 101-6.

[13] Mueckler, M. and Makepeace, C. (2006). Transmembrane segment 12 of the Glut1 glucose transporter is an outer helix and is not directly involved in the transport mechanism. *J. Biol. Chem.* 281, 36993-8.

[14] Marger, M.D. and Saier, M.H.,Jr. (1993). A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.* 18, 13-20.

[15] Joost, H.G., Bell, G.I., Best, J.D., Birnbaum, M.J., Charron, M.J., Chen, Y.T., Doege, H., James, D.E., Lodish, H.F., Moley, K.H., Moley, J.F., Mueckler, M., Rogers, S., Schurmann, A., Seino, S., and Thorens, B. (2002). Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. *Am. J. Physiol. Endocrinol. Metab.* 282, E974-6.

[16] Uldry, M. and Thorens, B. (2004). The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch.* 447, 480-9.

[17] Wu, X. and Freeze, H.H. (2002). GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80, 553-7.

[18] Manolescu, A.R., Witkowska, K., Kinnaird, A., Cessford, T., and Cheeseman, C. (2007). Facilitated hexose transporters: new perspectives on form and function. *Physiology (Bethesda)* 22, 234-40.

[19] Thorens, B. and Mueckler, M.M. (2009). Glucose transporters in the 21st Century. *Am. J. Physiol. Endocrinol. Metab.* 298: E141-5.

[20] Gould, G.W., Thomas, H.M., Jess, T.J., and Bell, G.I. (1991). Expression of human glucose transporters in Xenopus oocytes: kinetic characterization and substrate specificities of the erythrocyte, liver, and brain isoforms. *Biochemistry* 30, 5139-45.

[21] Evans, S.A., Doblado, M., Chi, M.M., Corbett, J.A., and Moley, K.H. (2009). Facilitative glucose transporter 9 expression affects glucose sensing in pancreatic beta-cells. *Endocrinology* 150, 5302-10.

[22] Phay, J.E., Hussain, H.B., and Moley, J.F. (2000). Cloning and expression analysis of a novel member of the facilitative glucose transporter family, SLC2A9 (GLUT9). *Genomics* 66, 217-20.

[23] Vitart, V., Rudan, I., Hayward, C., Gray, N.K., Floyd, J., Palmer, C.N., Knott, S.A., Kolcic, I., Polasek, O., Graessler, J., Wilson, J.F., Marinaki, A., Riches, P.L., Shu, X., Janicijevic, B., Smolej-Narancic, N., Gorgoni, B., Morgan, J., Campbell, S., Biloglav, Z., Barac-Lauc, L., Pericic, M., Klaric, I.M., Zgaga, L., Skaric-Juric, T., Wild, S.H.,

Richardson, W.A., Hohenstein, P., Kimber, C.H., Tenesa, A., Donnelly, L.A., Fairbanks, L.D., Aringer, M., McKeigue, P.M., Ralston, S.H., Morris, A.D., Rudan, P., Hastie, N.D., Campbell, H., and Wright, A.F. (2008). SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout. *Nat. Genet.* 40, 437-42.

[24] Caulfield, M.J., Munroe, P.B., O'Neill, D., Witkowska, K., Charchar, F.J., Doblado, M., Evans, S., Eyheramendy, S., Onipinla, A., Howard, P., Shaw-Hawkins, S., Dobson, R.J., Wallace, C., Newhouse, S.J., Brown, M., Connell, J.M., Dominiczak, A., Farrall, M., Lathrop, G.M., Samani, N.J., Kumari, M., Marmot, M., Brunner, E., Chambers, J., Elliott, P., Kooner, J., Laan, M., Org, E., Veldre, G., Viigimaa, M., Cappuccio, F.P., Ji, C., Iacone, R., Strazzullo, P., Moley, K.H., and Cheeseman, C. (2008). SLC2A9 is a high-capacity urate transporter in humans. *PLoS Med.* 5, e197.

[25] Doblado, M. and Moley, K.H. (2009). Facilitative glucose transporter 9, a unique hexose and urate transporter. *Am. J. Physiol. Endocrinol. Metab.* 297, E831-5.

[26] Corpe, C.P., Basaleh, M.M., Affleck, J., Gould, G., Jess, T.J., and Kellett, G.L. (1996). The regulation of GLUT5 and GLUT2 activity in the adaptation of intestinal brush-border fructose transport in diabetes. *Pflugers Arch.* 432, 192-201.

[27] Kellett, G.L. and Helliwell, P.A. (2000). The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. *Biochem. J.* 350 Pt 1, 155-62.

[28] Helliwell, P.A., Richardson, M., Affleck, J., and Kellett, G.L. (2000). Stimulation of fructose transport across the intestinal brush-border membrane by PMA is mediated by GLUT2 and dynamically regulated by protein kinase C. *Biochem. J.* 350 Pt 1, 149-54.

[29] Freeman, S.L., Bohan, D., Darcel, N., and Raybould, H.E. (2006). Luminal glucose sensing in the rat intestine has characteristics of a sodium-glucose cotransporter. *Am. J. Physiol. Gastrointest. Liver Physiol.* 291, G439-45.

[30] Cheeseman, C.I. and Tsang, R. (1996). The effect of GIP and glucagon-like peptides on intestinal basolateral membrane hexose transport. *Am. J. Physiol.* 271, G477-82.

[31] Cheeseman, C.I. (1997). Upregulation of SGLT-1 transport activity in rat jejunum induced by GLP-2 infusion in vivo. *Am. J. Physiol.* 273, R1965-71.

[32] Cheeseman, C. (2008). GLUT7: a new intestinal facilitated hexose transporter. *Am. J. Physiol. Endocrinol. Metab.* 295, E238-41.

[33] Li, Q., Manolescu, A., Ritzel, M., Yao, S., Slugoski, M., Young, J.D., Chen, X.Z., and Cheeseman, C.I. (2004). Cloning and functional characterization of the human GLUT7 isoform SLC2A7 from the small intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287, G236-42.

[34] Thorens, B. (1996). Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. *Am. J. Physiol.* 270, G541-53.

[35] Santer, R., Schneppenheim, R., Dombrowski, A., Gotze, H., Steinmann, B., and Schaub, J. (1997). Mutations in GLUT2, the gene for the liver-type glucose transporter, in patients with Fanconi-Bickel syndrome. *Nat. Genet.* 17, 324-6.

[36] Thorens, B., Guillam, M.T., Beermann, F., Burcelin, R., and Jaquet, M. (2000). Transgenic reexpression of GLUT1 or GLUT2 in pancreatic beta cells rescues GLUT2-

null mice from early death and restores normal glucose-stimulated insulin secretion. *J. Biol. Chem.* 275, 23751-8.

[37] Stumpel, F., Burcelin, R., Jungermann, K., and Thorens, B. (2001). Normal kinetics of intestinal glucose absorption in the absence of GLUT2: evidence for a transport pathway requiring glucose phosphorylation and transfer into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11330-5.

[38] Stumpel, F., Burcelin, R., Jungermann, K., and Thorens, B. (2001). Normal kinetics of intestinal glucose absorption in the absence of GLUT2: evidence for a transport pathway requiring glucose phosphorylation and transfer into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11330-5.

[39] Mueckler, M. (1994). Facilitative glucose transporters. *Eur. J. Biochem.* 219, 713-25.

[40] Gonzalez, G.A. and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59, 675-80.

[41] Herzig, S., Long, F., Jhala, U.S., Hedrick, S., Quinn, R., Bauer, A., Rudolph, D., Schutz, G., Yoon, C., Puigserver, P., Spiegelman, B., and Montminy, M. (2001). CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 413, 179-83.

[42] Gross, D.N., van den Heuvel, A.P., and Birnbaum, M.J. (2008). The role of FoxO in the regulation of metabolism. *Oncogene* 27, 2320-36.

[43] Silverman, M. (1991). Structure and function of hexose transporters. *Annu. Rev. Biochem.* 60, 757-94.

[44] DeFronzo, R.A., Gunnarsson, R., Bjorkman, O., Olsson, M., and Wahren, J. (1985). Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J. Clin. Invest.* 76, 149-55.

[45] Livingstone, C., Thomson, F.J., Arbuckle, M.I., Campbell, I.W., Jess, T.J., Kane, S., Moyes, C., Porter, L.M., Rice, J.E., Seatter, M.J., and Gould, G.W. (1996). Hormonal regulation of the insulin-responsive glucose transporter, GLUT4: some recent advances. *Proc. Nutr. Soc.* 55, 179-90.

[46] Zaid, H., Antonescu, C.N., Randhawa, V.K., and Klip, A. (2008). Insulin action on glucose transporters through molecular switches, tracks and tethers. *Biochem. J.* 413, 201-15.

[47] Huang, C., Thirone, A.C., Huang, X., and Klip, A. (2005). Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in l6 myotubes. *J. Biol. Chem.* 280, 19426-35.

[48] Bouzakri, K., Zachrisson, A., Al-Khalili, L., Zhang, B.B., Koistinen, H.A., Krook, A., and Zierath, J.R. (2006). siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. *Cell. Metab.* 4, 89-96.

[49] Ishiki, M., Randhawa, V.K., Poon, V., Jebailey, L., and Klip, A. (2005). Insulin regulates the membrane arrival, fusion, and C-terminal unmasking of glucose transporter-4 via distinct phosphoinositides. *J. Biol. Chem.* 280, 28792-802.

[50] Wang, Q., Somwar, R., Bilan, P.J., Liu, Z., Jin, J., Woodgett, J.R., and Klip, A. (1999). Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* 19, 4008-18.

[51] Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B.,3rd, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., and Birnbaum, M.J. (2001). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292, 1728-31.

[52] Jiang, Z.Y., Zhou, Q.L., Coleman, K.A., Chouinard, M., Boese, Q., and Czech, M.P. (2003). Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7569-74.

[53] Katome, T., Obata, T., Matsushima, R., Masuyama, N., Cantley, L.C., Gotoh, Y., Kishi, K., Shiota, H., and Ebina, Y. (2003). Use of RNA interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of AKT/protein kinase B isoforms in insulin actions. *J. Biol. Chem.* 278, 28312-23.

[54] Ng, Y., Ramm, G., Lopez, J.A., and James, D.E. (2008). Rapid activation of Akt2 is sufficient to stimulate GLUT4 translocation in 3T3-L1 adipocytes. *Cell. Metab.* 7, 348-56.

[55] Kohn, A.D., Summers, S.A., Birnbaum, M.J., and Roth, R.A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.* 271, 31372-8.

[56] Larance, M., Ramm, G., Stockli, J., van Dam, E.M., Winata, S., Wasinger, V., Simpson, F., Graham, M., Junutula, J.R., Guilhaus, M., and James, D.E. (2005).

Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. *J. Biol. Chem.* 280, 37803-13.

[57] Eguez, L., Lee, A., Chavez, J.A., Miinea, C.P., Kane, S., Lienhard, G.E., and McGraw, T.E. (2005). Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell. Metab.* 2, 263-72.

[58] Sano, H., Kane, S., Sano, E., Miinea, C.P., Asara, J.M., Lane, W.S., Garner, C.W., and Lienhard, G.E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J. Biol. Chem.* 278, 14599-602.

[59] Zeigerer, A., McBrayer, M.K., and McGraw, T.E. (2004). Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160. *Mol. Biol. Cell* 15, 4406-15.

[60] Sakamoto, K. and Holman, G.D. (2008). Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *Am. J. Physiol. Endocrinol. Metab.* 295, E29-37.

[61] Omata, W., Shibata, H., Li, L., Takata, K., and Kojima, I. (2000). Actin filaments play a critical role in insulin-induced exocytotic recruitment but not in endocytosis of GLUT4 in isolated rat adipocytes. *Biochem. J.* 346 Pt 2, 321-8.

[62] Wang, Q., Bilan, P.J., Tsakiridis, T., Hinek, A., and Klip, A. (1998). Actin filaments participate in the relocalization of phosphatidylinositol3-kinase to glucose transporter-containing compartments and in the stimulation of glucose uptake in 3T3-L1 adipocytes. *Biochem. J.* 331 ( Pt 3), 917-28.

[63] Brozinick, J.T.,Jr, Hawkins, E.D., Strawbridge, A.B., and Elmendorf, J.S. (2004). Disruption of cortical actin in skeletal muscle demonstrates an essential role of the cytoskeleton in glucose transporter 4 translocation in insulin-sensitive tissues. *J. Biol. Chem.* 279, 40699-706.

[64] Tong, P., Khayat, Z.A., Huang, C., Patel, N., Ueyama, A., and Klip, A. (2001). Insulin-induced cortical actin remodeling promotes GLUT4 insertion at muscle cell membrane ruffles. *J. Clin. Invest.* 108, 371-81.

[65] Kanzaki, M. and Pessin, J.E. (2001). Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. *J. Biol. Chem.* 276, 42436-44.

[66] Strawbridge, A.B. and Elmendorf, J.S. (2005). Phosphatidylinositol 4,5-bisphosphate reverses endothelin-1-induced insulin resistance via an actin-dependent mechanism. *Diabetes* 54, 1698-705.

[67] World Health Organization. (2012). Diabetes. http://www.who.int/mediacentre/factsheets/fs312/en/index.html. Accessed July 15th, 2012.

[68] National Diabetes Surveillance System. (2009). Report from the National Diabetes Surveillance System: Diabetes in Canada, 2009, 1-28.

[69] Pauls, F. and Drury, D.R. (1942). The rate of glucose absorption from the intestine of diabetic rats . *Am J Physiol* 137, 242-5.

[70] Crane, R.K. (1961). An effect of alloxan-diabetes on the active transport of sugars by rat small intestine, in vitro. *Biochem. Biophys. Res. Commun.* 4, 436-40.

[71] Olsen, W.A. and Rosenberg, I.H. (1970). Intestinal transport of sugars and amino acids in diabetic rats. *J. Clin. Invest.* 49, 96-105.

[72] Schedl, H.P. and Wilson, H.D. (1971). Effects of diabetes on intestinal growth and hexose transport in the rat. *Am. J. Physiol.* 220, 1739-45.

[73] Csaky, T.Z. and Fischer, E. (1981). Intestinal sugar transport in experimental diabetes. *Diabetes* 30, 568-74.

[74] Hopfer, U. (1975). Diabetes mellitus: changes in the transport properties of isolated intestinal microvillous membranes. *Proc. Natl. Acad. Sci. U. S. A.* 72, 2027-31.

[75] Ramaswamy, K., Peterson, M.A., Flint, P.W., and Whalen, G.E. (1980). Transport of monosaccharides by the small intestine of genetically diabetic mice. *Gastroenterology* 78, 464-9.

[76] Lorenz-Meyer, H., Thiel, F., Menge, H., Gottesburen, H., and Riecken, E.O. (1977). Structural and functional studies on the transformation of the intestinal mucosa in rats with experimental diabetes. *Res. Exp. Med. (Berl)* 170, 89-99.

[77] Debnam, E.S., Ebrahim, H.Y., and Swaine, D.J. (1990). Diabetes mellitus and sugar transport across the brush-border and basolateral membranes of rat jejunal enterocytes. *J. Physiol.* 424, 13-25.

[78] Burant, C.F., Flink, S., DePaoli, A.M., Chen, J., Lee, W.S., Hediger, M.A., Buse, J.B., and Chang, E.B. (1994). Small intestine hexose transport in experimental diabetes. Increased transporter mRNA and protein expression in enterocytes. *J. Clin. Invest.* 93, 578-85.

[79] Corpe, C., Sreenan, S., and Burant, C. (2001). Effects of type-2 diabetes and troglitazone on the expression patterns of small intestinal sugar transporters and PPAR-gamma in the Zucker diabetic fatty rat. *Digestion* 63, 116-23.

[80] Dyer, J., Wood, I.S., Palejwala, A., Ellis, A., and Shirazi-Beechey, S.P. (2002). Expression of monosaccharide transporters in intestine of diabetic humans. *Am. J. Physiol. Gastrointest. Liver Physiol.* 282, G241-8.

[81] Madsen, K.L., Ariano, D., and Fedorak, R.N. (1996). Insulin downregulates diabetic-enhanced intestinal glucose transport rapidly in ileum and slowly in jejunum. *Can. J. Physiol. Pharmacol.* 74, 1294-301.

[82] Fedorak, R.N., Chang, E.B., Madara, J.L., and Field, M. (1987). Intestinal adaptation to diabetes. Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. *J. Clin. Invest.* 79, 1571-8.

[83] Fujii, Y., Kaizuka, M., Hashida, F., Maruo, J., Sato, E., Yasuda, H., Kurokawa, T., and Ishibashi, S. (1991). Insulin regulates Na+/glucose cotransporter activity in rat small intestine. *Biochim. Biophys. Acta* 1063, 90-4.

[84] Pennington, A.M., Corpe, C.P., and Kellett, G.L. (1994). Rapid regulation of rat jejunal glucose transport by insulin in a luminally and vascularly perfused preparation. *J. Physiol.* 478 ( Pt 2), 187-93.

[85] Bergeron, R., Previs, S.F., Cline, G.W., Perret, P., Russell, R.R.,3rd, Young, L.H., and Shulman, G.I. (2001). Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* 50, 1076-82.

[86] Tobin, V., Le Gall, M., Fioramonti, X., Stolarczyk, E., Blazquez, A.G., Klein, C., Prigent, M., Serradas, P., Cuif, M.H., Magnan, C., Leturque, A., and Brot-Laroche, E. (2008). Insulin internalizes GLUT2 in the enterocytes of healthy but not insulin-resistant mice. *Diabetes* 57, 555-62.

[87] Garvey, W.T., Hueckstead, T.P., Matthaei, S., and Olefsky, J.M. (1988). Role of glucose transporters in the cellular insulin resistance of type II non-insulin-dependent diabetes mellitus. *J. Clin. Invest.* 81, 1528-36.

[88] Garvey, W.T., Maianu, L., Hueckstead, T.P., Birnbaum, M.J., Molina, J.M., and Ciaraldi, T.P. (1991). Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. *J. Clin. Invest.* 87, 1072-81.

[89] Kozka, I.J., Clark, A.E., and Holman, G.D. (1991). Chronic treatment with insulin selectively down-regulates cell-surface GLUT4 glucose transporters in 3T3-L1 adipocytes. *J. Biol. Chem.* 266, 11726-31.

[90] Berger, J., Biswas, C., Vicario, P.P., Strout, H.V., Saperstein, R., and Pilch, P.F. (1989). Decreased expression of the insulin-responsive glucose transporter in diabetes and fasting. *Nature* 340, 70-2.

[91] Sivitz, W.I., DeSautel, S.L., Kayano, T., Bell, G.I., and Pessin, J.E. (1989). Regulation of glucose transporter messenger RNA in insulin-deficient states. *Nature* 340, 72-4.

[92] Calderhead, D.M., Kitagawa, K., Tanner, L.I., Holman, G.D., and Lienhard, G.E. (1990). Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. *J. Biol. Chem.* 265, 13801-8.

[93] Calderhead, D.M. and Lienhard, G.E. (1988). Labeling of glucose transporters at the cell surface in 3T3-L1 adipocytes. Evidence for both translocation and a second mechanism in the insulin stimulation of transport. *J. Biol. Chem.* 263, 12171-4.

[94] Le Marchand-Brustel, Y., Tanti, J.F., Cormont, M., Ricort, J.M., Gremeaux, T., and Grillo, S. (1999). From insulin receptor signalling to Glut 4 translocation abnormalities in obesity and insulin resistance. *J. Recept. Signal Transduct. Res.* 19, 217-28.

[95] Smith, U. (2002). Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance--is insulin resistance initiated in the adipose tissue? *Int. J. Obes. Relat. Metab. Disord.* 26, 897-904.

[96] Sesti, G. (2006). Pathophysiology of insulin resistance. *Best Pract. Res. Clin. Endocrinol. Metab.* 20, 665-79.

[97] Flores-Riveros, J.R., McLenithan, J.C., Ezaki, O., and Lane, M.D. (1993). Insulin down-regulates expression of the insulin-responsive glucose transporter (GLUT4) gene: effects on transcription and mRNA turnover. *Proc. Natl. Acad. Sci. U. S. A.* 90, 512-6.

[98] Ricort, J.M., Tanti, J.F., Van Obberghen, E., and Le Marchand-Brustel, Y. (1995). Alterations in insulin signalling pathway induced by prolonged insulin treatment of 3T3-L1 adipocytes. *Diabetologia* 38, 1148-56.

[99] Chen, G., Raman, P., Bhonagiri, P., Strawbridge, A.B., Pattar, G.R., and Elmendorf, J.S. (2004). Protective effect of phosphatidylinositol 4,5-bisphosphate against cortical

filamentous actin loss and insulin resistance induced by sustained exposure of 3T3-L1 adipocytes to insulin. *J. Biol. Chem.* 279, 39705-9.

[100] Ross, S.A., Chen, X., Hope, H.R., Sun, S., McMahon, E.G., Broschat, K., and Gulve, E.A. (2000). Development and comparison of two 3T3-L1 adipocyte models of insulin resistance: increased glucose flux vs glucosamine treatment. *Biochem. Biophys. Res. Commun.* 273, 1033-41.

[101] Garvey, W.T., Huecksteadt, T.P., and Birnbaum, M.J. (1989). Pretranslational suppression of an insulin-responsive glucose transporter in rats with diabetes mellitus. *Science* 245, 60-3.

[102] King, P.A., Horton, E.D., Hirshman, M.F., and Horton, E.S. (1992). Insulin resistance in obese Zucker rat (fa/fa) skeletal muscle is associated with a failure of glucose transporter translocation. *J. Clin. Invest.* 90, 1568-75.

[103] Dohm, G.L., Tapscott, E.B., Pories, W.J., Dabbs, D.J., Flickinger, E.G., Meelheim, D., Fushiki, T., Atkinson, S.M., Elton, C.W., and Caro, J.F. (1988). An in vitro human muscle preparation suitable for metabolic studies. Decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. *J. Clin. Invest.* 82, 486-94.

[104] Ramlal, T., Rastogi, S., Vranic, M., and Klip, A. (1989). Decrease in glucose transporter number in skeletal muscle of mildly diabetic (streptozotocin-treated) rats. *Endocrinology* 125, 890-7.

[105] Koranyi, L., James, D., Mueckler, M., and Permutt, M.A. (1990). Glucose transporter levels in spontaneously obese (db/db) insulin-resistant mice. *J. Clin. Invest.* 85, 962-7.

[106] Kahn, B.B., Rossetti, L., Lodish, H.F., and Charron, M.J. (1991). Decreased in vivo glucose uptake but normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats. *J. Clin. Invest.* 87, 2197-206.

[107] Pedersen, O., Bak, J.F., Andersen, P.H., Lund, S., Moller, D.E., Flier, J.S., and Kahn, B.B. (1990). Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* 39, 865-70.

[108] Henriksen, E.J., Bourey, R.E., Rodnick, K.J., Koranyi, L., Permutt, M.A., and Holloszy, J.O. (1990). Glucose transporter protein content and glucose transport capacity in rat skeletal muscles. *Am. J. Physiol.* 259, E593-8.

[109] Richardson, J.M., Balon, T.W., Treadway, J.L., and Pessin, J.E. (1991). Differential regulation of glucose transporter activity and expression in red and white skeletal muscle. *J. Biol. Chem.* 266, 12690-4.

[110] Hardin, D.S., Dominguez, J.H., and Garvey, W.T. (1993). Muscle group-specific regulation of GLUT 4 glucose transporters in control, diabetic, and insulin-treated diabetic rats. *Metabolism* 42, 1310-5.

[111] Huang, C., Somwar, R., Patel, N., Niu, W., Torok, D., and Klip, A. (2002). Sustained exposure of L6 myotubes to high glucose and insulin decreases insulin-stimulated GLUT4 translocation but upregulates GLUT4 activity. *Diabetes* 51, 2090-8.

[112] Pirola, L., Bonnafous, S., Johnston, A.M., Chaussade, C., Portis, F., and Van Obberghen, E. (2003). Phosphoinositide 3-kinase-mediated reduction of insulin receptor substrate-1/2 protein expression via different mechanisms contributes to the insulin-induced desensitization of its signaling pathways in L6 muscle cells. *J. Biol. Chem.* 278, 15641-51.

[113] McCarthy, A.M., Spisak, K.O., Brozinick, J.T., and Elmendorf, J.S. (2006). Loss of cortical actin filaments in insulin-resistant skeletal muscle cells impairs GLUT4 vesicle trafficking and glucose transport. *Am. J. Physiol. Cell. Physiol.* 291, C860-8.

[114] Creutzfeldt, W. and Ebert, R. (1985). New developments in the incretin concept. *Diabetologia* 28, 565-73.

[115] Kieffer, T.J. and Habener, J.F. (1999). The glucagon-like peptides. *Endocr. Rev.* 20, 876-913.

[116] Kim, S.J., Choi, W.S., Han, J.S., Warnock, G., Fedida, D., and McIntosh, C.H. (2005). A novel mechanism for the suppression of a voltage-gated potassium channel by glucose-dependent insulinotropic polypeptide: protein kinase A-dependent endocytosis. *J. Biol. Chem.* 280, 28692-700.

[117] Diab, D.L. and D'Alessio, D.A. (2010). The contribution of enteroinsular hormones to the pathogenesis of type 2 diabetes mellitus. *Curr. Diab Rep.* 10, 192-8.

[118] Nauck, M., Stockmann, F., Ebert, R., and Creutzfeldt, W. (1986). Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* 29, 46-52.

[119] Muscelli, E., Mari, A., Casolaro, A., Camastra, S., Seghieri, G., Gastaldelli, A., Holst, J.J., and Ferrannini, E. (2008). Separate impact of obesity and glucose tolerance

on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* 57, 1340-8.

[120] Vilsboll, T., Krarup, T., Deacon, C.F., Madsbad, S., and Holst, J.J. (2001). Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. *Diabetes* 50, 609-13.

[121] Hojberg, P.V., Zander, M., Vilsboll, T., Knop, F.K., Krarup, T., Volund, A., Holst, J.J., and Madsbad, S. (2008). Near normalisation of blood glucose improves the potentiating effect of GLP-1 on glucose-induced insulin secretion in patients with type 2 diabetes. *Diabetologia* 51, 632-40.

[122] Salehi, M., Aulinger, B., Prigeon, R.L., and D'Alessio, D.A. (2010). Effect of endogenous GLP-1 on insulin secretion in type 2 diabetes. *Diabetes* 59, 1330-7.

[123] Katsuura, G., Asakawa, A., and Inui, A. (2002). Roles of pancreatic polypeptide in regulation of food intake. *Peptides* 23, 323-9.

[124] Simpson, K., Parker, J., Plumer, J., and Bloom, S. (2012). CCK, PYY and PP: the control of energy balance. *Handb. Exp. Pharmacol.* (209), 209-30.

[125] Kojima, S., Ueno, N., Asakawa, A., Sagiyama, K., Naruo, T., Mizuno, S., and Inui, A. (2007). A role for pancreatic polypeptide in feeding and body weight regulation. *Peptides* 28, 459-63.

[126] Asakawa, A., Inui, A., Ueno, N., Fujimiya, M., Fujino, M.A., and Kasuga, M. (1999). Mouse pancreatic polypeptide modulates food intake, while not influencing anxiety in mice. *Peptides* 20, 1445-8.

[127] Batterham, R.L., Le Roux, C.W., Cohen, M.A., Park, A.J., Ellis, S.M., Patterson, M., Frost, G.S., Ghatei, M.A., and Bloom, S.R. (2003). Pancreatic polypeptide reduces appetite and food intake in humans. *J. Clin. Endocrinol. Metab.* 88, 3989-92.

[128] Parker, R.M. and Herzog, H. (1999). Regional distribution of Y-receptor subtype mRNAs in rat brain. *Eur. J. Neurosci.* 11, 1431-48.

[129] Howarth, F.C., Al Kitbi, M.K., Hameed, R.S., and Adeghate, E. (2011). Pancreatic peptides in young and elderly Zucker type 2 diabetic fatty rats. *JOP* 12, 567-73.

[130] Weyer, C., Salbe, A.D., Lindsay, R.S., Pratley, R.E., Bogardus, C., and Tataranni, P.A. (2001). Exaggerated pancreatic polypeptide secretion in Pima Indians: can an increased parasympathetic drive to the pancreas contribute to hyperinsulinemia, obesity, and diabetes in humans? *Metabolism* 50, 223-30.

[131] Kahleova, H., Mari, A., Nofrate, V., Matoulek, M., Kazdova, L., Hill, M., and Pelikanova, T. (2012). Improvement in beta-cell function after diet-induced weight loss is associated with decrease in pancreatic polypeptide in subjects with type 2 diabetes. *J. Diabetes Complications.* 26, 442-9.

[132] Grandt, D., Schimiczek, M., Beglinger, C., Layer, P., Goebell, H., Eysselein, V.E., and Reeve, J.R.,Jr. (1994). Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. *Regul. Pept.* 51, 151-9.

[133] Adrian, T.E., Ferri, G.L., Bacarese-Hamilton, A.J., Fuessl, H.S., Polak, J.M., and Bloom, S.R. (1985). Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89, 1070-7.

[134] Batterham, R.L., Heffron, H., Kapoor, S., Chivers, J.E., Chandarana, K., Herzog, H., Le Roux, C.W., Thomas, E.L., Bell, J.D., and Withers, D.J. (2006). Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell. Metab.* 4, 223-33.

[135] Helou, N., Obeid, O., Azar, S.T., and Hwalla, N. (2008). Variation of postprandial PYY 3-36 response following ingestion of differing macronutrient meals in obese females. *Ann. Nutr. Metab.* 52, 188-95.

[136] Chandarana, K., Drew, M.E., Emmanuel, J., Karra, E., Gelegen, C., Chan, P., Cron, N.J., and Batterham, R.L. (2009). Subject standardization, acclimatization, and sample processing affect gut hormone levels and appetite in humans. *Gastroenterology* 136, 2115-26.

[137] Adrian, T.E., Ferri, G.L., Bacarese-Hamilton, A.J., Fuessl, H.S., Polak, J.M., and Bloom, S.R. (1985). Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89, 1070-7.

[138] van den Hoek, A.M., Heijboer, A.C., Corssmit, E.P., Voshol, P.J., Romijn, J.A., Havekes, L.M., and Pijl, H. (2004). PYY3-36 reinforces insulin action on glucose disposal in mice fed a high-fat diet. *Diabetes* 53, 1949-52.

[139] Pittner, R.A., Moore, C.X., Bhavsar, S.P., Gedulin, B.R., Smith, P.A., Jodka, C.M., Parkes, D.G., Paterniti, J.R., Srivastava, V.P., and Young, A.A. (2004). Effects of PYY[3-36] in rodent models of diabetes and obesity. *Int. J. Obes. Relat. Metab. Disord.* 28, 963-71.

[140] Vrang, N., Madsen, A.N., Tang-Christensen, M., Hansen, G., and Larsen, P.J. (2006). PYY(3-36) reduces food intake and body weight and improves insulin sensitivity in rodent models of diet-induced obesity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R367-75.

[141] Ukkola, O.H., Puurunen, V.P., Piira, O.P., Niva, J.T., Lepojarvi, E.S., Tulppo, M.P., and Huikuri, H.V. (2011). High serum fasting peptide YY (3-36) is associated with obesity-associated insulin resistance and type 2 diabetes. *Regul. Pept.* 170, 38-42.

[142] Ukkola, O.H., Puurunen, V.P., Piira, O.P., Niva, J.T., Lepojarvi, E.S., Tulppo, M.P., and Huikuri, H.V. (2011). High serum fasting peptide YY (3-36) is associated with obesity-associated insulin resistance and type 2 diabetes. *Regul. Pept.* 170, 38-42.

[143] Viardot, A., Heilbronn, L.K., Herzog, H., Gregersen, S., and Campbell, L.V. (2008). Abnormal postprandial PYY response in insulin sensitive nondiabetic subjects with a strong family history of type 2 diabetes. *Int. J. Obes. (Lond)* 32, 943-8.

[144] Torekov, S.S., Larsen, L.H., Glumer, C., Borch-Johnsen, K., Jorgensen, T., Holst, J.J., Madsen, O.D., Hansen, T., and Pedersen, O. (2005). Evidence of an association between the Arg72 allele of the peptide YY and increased risk of type 2 diabetes. *Diabetes* 54, 2261-5.

[145] Date, Y., Murakami, N., Toshinai, K., Matsukura, S., Niijima, A., Matsuo, H., Kangawa, K., and Nakazato, M. (2002). The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 123, 1120-8.

[146] Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402, 656-60.

[147] Wren, A.M., Seal, L.J., Cohen, M.A., Brynes, A.E., Frost, G.S., Murphy, K.G., Dhillo, W.S., Ghatei, M.A., and Bloom, S.R. (2001). Ghrelin enhances appetite and increases food intake in humans. *J. Clin. Endocrinol. Metab.* 86, 5992.

[148] Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature* 409, 194-8.

[149] Wren, A.M., Small, C.J., Ward, H.L., Murphy, K.G., Dakin, C.L., Taheri, S., Kennedy, A.R., Roberts, G.H., Morgan, D.G., Ghatei, M.A., and Bloom, S.R. (2000). The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 141, 4325-8.

[150] Druce, M.R., Wren, A.M., Park, A.J., Milton, J.E., Patterson, M., Frost, G., Ghatei, M.A., Small, C., and Bloom, S.R. (2005). Ghrelin increases food intake in obese as well as lean subjects. *Int. J. Obes. (Lond)* 29, 1130-6.

[151] Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., and Weigle, D.S. (2001). A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 50, 1714-9.

[152] Hansen, T.K., Dall, R., Hosoda, H., Kojima, M., Kangawa, K., Christiansen, J.S., and Jorgensen, J.O. (2002). Weight loss increases circulating levels of ghrelin in human obesity. *Clin. Endocrinol. (Oxf)* 56, 203-6.

[153] English, P.J., Ghatei, M.A., Malik, I.A., Bloom, S.R., and Wilding, J.P. (2002). Food fails to suppress ghrelin levels in obese humans. *J. Clin. Endocrinol. Metab.* 87, 2984.

[154] Poykko, S.M., Kellokoski, E., Horkko, S., Kauma, H., Kesaniemi, Y.A., and Ukkola, O. (2003). Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes* 52, 2546-53.

[155] McLaughlin, T., Abbasi, F., Lamendola, C., Frayo, R.S., and Cummings, D.E. (2004). Plasma ghrelin concentrations are decreased in insulin-resistant obese adults relative to equally obese insulin-sensitive controls. *J. Clin. Endocrinol. Metab.* 89, 1630-5.

[156] Barazzoni, R., Zanetti, M., Ferreira, C., Vinci, P., Pirulli, A., Mucci, M., Dore, F., Fonda, M., Ciocchi, B., Cattin, L., and Guarnieri, G. (2007). Relationships between desacylated and acylated ghrelin and insulin sensitivity in the metabolic syndrome. *J. Clin. Endocrinol. Metab.* 92, 3935-40.

[157] Schiel, R., Voigt, U., Ross, I.S., Braun, A., Rillig, A., Hunger-Dathe, W., Stein, G., and Muller, U.A. (2006). Structured diabetes therapy and education improves the outcome of patients with insulin treated diabetes mellitus. The 10 year follow-up of a prospective, population-based survey on the quality of diabetes care (the JEVIN Trial). *Exp. Clin. Endocrinol. Diabetes* 114, 18-27.

[158] Harris, M.I. and National Health and Nutrition Examination Survey (NHANES III). (2001). Frequency of blood glucose monitoring in relation to glycemic control in patients with type 2 diabetes. *Diabetes Care* 24, 979-82.

[159] Bhattacharyya, O.K., Estey, E.A., Cheng, A.Y., and Canadian Diabetes Association 2008. (2009). Update on the Canadian Diabetes Association 2008 clinical practice guidelines. *Can. Fam. Physician* 55, 39-43.

[160] American Diabetes Association. (2012). Standards of medical care in diabetes--2012. *Diabetes Care* 35 Suppl 1, S11-63.

[161] Lochhead, P.A., Salt, I.P., Walker, K.S., Hardie, D.G., and Sutherland, C. (2000). 5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes* 49, 896-903.

[162] Rendell, M. (2004). The role of sulphonylureas in the management of type 2 diabetes mellitus. *Drugs* 64, 1339-58.

[163] Ismail-Beigi, F. (2012). Clinical practice. Glycemic management of type 2 diabetes mellitus. *N. Engl. J. Med.* 366, 1319-27.

[164] Barnett, A., Allsworth, J., Jameson, K., and Mann, R. (2007). A review of the effects of antihyperglycaemic agents on body weight: the potential of incretin targeted therapies. *Curr. Med. Res. Opin.* 23, 1493-507.

[165] Bailey, C.J., Iqbal, N., T'joen, C., and List, J.F. (2012). Dapagliflozin monotherapy in drug-naive patients with diabetes: a randomized-controlled trial of low-dose range(dagger). *Diabetes Obes. Metab.* 9999, 10.1111/j.1463,1326.2012.01659.x.

[166] The Canadian Special Crops Association. (2008). Buckwheat-Information. http://www.specialcrops.mb.ca/crops/buckwheat.html. Accessed September 25th, 2012.

[167] Agriculture and Agri-Food Canada. Canadian Buckwheat Statistics. (2012). http://www4.agr.gc.ca/AAFC-AAC/display-afficher.do?id=1174581273612&lang=eng#stat. Accessed: 25th September, 2012.

[168] Pomeranz, Y. (1983). Buckwheat: structure, composition, and utilization. *Crit. Rev. Food Sci. Nutr.* 19, 213-58.

[169] Vojtíšková, P., Kmentová, K., Kubáň, V., and Kráčmar, S. (2012). Chemical composition of buckwheat plant (Fagopyrum esculentum) and selected buckwheat products. *J Micro Bio Food Sci* 1, 1011-9.

[170] Zheng, G., Sosulski, G., and Tyler, R. (1988). Wet-milling, composition and functional properties of starch and protein isolated from buckwheat groats. *Food Research International, 30*, 493-502. *Food Res Int* 30, 493-502.

[171] Skrabanja, V. and Kreft, I. (1998). Resistant starch formation following autoclaving of buckwheat (*Fagopyrum esculentum* Moech) groats. An in vitro study. *J. Agric. Food Chem.* 46, 2020-3.

[172] Pomeranz, Y. and Robbins, G.S. (1972). Amino acid composition of buckwheat. *J. Agric. Food Chem.* 20, 270-4.

[173] Farrell, D.L. (1978). A nutritional evaluation of buckwheat (*Fagopyrum esculentum*). *Animal Feed Sci Technol* 3, 95-108.

[174] Javournik, B., Eggum, B.O., and Kreft, I. (1981). Studies on protein fractions and protein quality of buckwheat. *Genetika* 13, 115-8.

[175] Ikeda, K., Oku, M. Kusano, T. & Yasumoto, K. (1986). Inhibitory potency of plant antinutrients towards the in vitro digestibility of buckwheat protein. *Journal of Food Science, 51*, 1527-1530. *J Food Sci* 51, 1527-30.

[176] Ikeda, K., Arai, R., and Kreft, I. (1998). A molecular basis for the textural characteristics of buckwheat products Manitoba. In *Advances in Buckwheat Research* (Campbell, C. and Przybylski R., eds.) pp. 57-60, Winnipeg, Manitoba.

[177] Steadman, K.J., Burgoon, M.S., Lewis, B.A., Edwardson, S.E., and Obendorf, R. (2001). Minerals, phytic acid, tannin and rutin in buckwheat seed milling fractions. *J Sci Food Agric* 81, 1094-100.

[178] Kreft, S., Knapp, M., and Kreft, I. (1999). Extraction of rutin from buckwheat (Fagopyrum esculentumMoench) seeds and determination by capillary electrophoresis. *J. Agric. Food Chem.* 47, 4649-52.

[179] Zhang, H.W., Zhang, Y.H., Lu, M.J., Tong, W.J., and Cao, G.W. (2007). Comparison of hypertension, dyslipidaemia and hyperglycaemia between buckwheat seed-consuming and non-consuming Mongolian-Chinese populations in Inner Mongolia, China. *Clin. Exp. Pharmacol. Physiol.* 34, 838-44.

[180] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia and hyperlipidemia. In: *Proceedings of the 5th International Symposium on Buckwheat*, Lin, R., Zhou, M., Tao, Y., Li, J., Zhang, Z., Eds.; Agriculture Publishing House: Beijing, China, 458-64.

[181] Wang, J., Liu, Z., Fu, X., and Run, M. (1992). A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: *Proceedings of the 5th International Symposium on Buckwheat,* Lin, R., Zhou, M., Tao, Y., Li, J., Zhang, Z., Eds.; Agriculture Publishing House: Beijing, China, 465-7.

[182] Tadera, K., Minami, Y., Takamatsu, K., and Matsuoka, T. (2006). Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *J. Nutr. Sci. Vitaminol. (Tokyo)* 52, 149-53.

[183] Hosaka, T., Nii, Y., Tomotake, H., Ito, T., Tamanaha, A., Yamasaka, Y., Sasaga, S., Edazawa, K., Tsutsumi, R., Shuto, E., Okahisa, N., Iwata, S., and Sakai, T. (2011). Extracts of common buckwheat bran prevent sucrose digestion. *J. Nutr. Sci. Vitaminol. (Tokyo)* 57, 441-5.

[184] Steadman, K.J., Burgoon, M.S., Schuster, R.L., Lewis, B.A., Edwardson, S.E., and Obendorf, R.L. (2000). Fagopyritols, D-chiro-inositol, and other soluble carbohydrates in buckwheat seed milling fractions. *J. Agric. Food Chem.* 48, 2843-7.

[185] Ostlund, R.E.,Jr, McGill, J.B., Herskowitz, I., Kipnis, D.M., Santiago, J.V., and Sherman, W.R. (1993). D-chiro-inositol metabolism in diabetes mellitus. *Proc. Natl. Acad. Sci. U. S. A.* 90, 9988-92.

[186] Ortmeyer, H.K., Huang, L.C., Zhang, L., Hansen, B.C., and Larner, J. (1993). Chiroinositol deficiency and insulin resistance. II. Acute effects of D-chiroinositol administration in streptozotocin-diabetic rats, normal rats given a glucose load, and spontaneously insulin-resistant rhesus monkeys. *Endocrinology* 132, 646-51.

[187] Brautigan, D.L., Brown, M., Grindrod, S., Chinigo, G., Kruszewski, A., Lukasik, S.M., Bushweller, J.H., Horal, M., Keller, S., Tamura, S., Heimark, D.B., Price, J., Larner, A.N., and Larner, J. (2005). Allosteric activation of protein phosphatase 2C by D-chiro-inositol-galactosamine, a putative mediator mimetic of insulin action. *Biochemistry* 44, 11067-73.

[188] Kawa, J.M., Przybylski, R., and Taylor, C.G. (2003). Urinary chiro-inositol and myo-inositol excretion is elevated in the diabetic db/db mouse and streptozotocin diabetic rat. *Exp. Biol. Med. (Maywood)* 228, 907-14.

[189] Yao, Y., Shan, F., Bian, J., Chen, F., Wang, M., and Ren, G. (2008). D-chiro-inositol-enriched tartary buckwheat bran extract lowers the blood glucose level in KK-Ay mice. *J. Agric. Food Chem.* 56, 10027-31.

[190] Curran, J.M., Stringer, D.M., Wright, B., Taylor, C.G., Przybylski, R., and Zahradka, P. (2010). Biological response of hepatomas to an extract of Fagopyrum esculentum M. (buckwheat) is not mediated by inositols or rutin. *J. Agric. Food Chem.* 58, 3197-204.

# Chapter 3: Rationale, Hypotheses, and Objectives

## 3.1 Rationale

T2DM is a chronic disease characterized by insulin resistance and hyperglycemia. Glucose-lowering therapies aimed at reducing HbA1c and the associated macro- and microvascular complications of uncontrolled hyperglycemia are the cornerstone of T2DM management. Several large-scale clinical intervention trials seeking to determine the efficacy of intensive pharmacological HbA1c-lowering therapy for reducing CVD risk have found that such intensive glucose-lowering therapy produces only modest reductions in CVD risk, but leads to significantly more hypoglycemic episodes and weight gain [1,2]. In addition, the increased availability of oral anti-hyperglycemic agents has not led to a corresponding reduction in the incidence and prevalence of T2DM. Furthermore, the use of such agents is often associated with several unpleasant side effects, including diarrhea, nausea, vomiting, hypoglycemia, edema, and weight gain [3]. Weight gain is particularly disconcerting, as often patients with T2DM are overweight or obese, and the mild to moderate weight gain associated with these oral anti-hyperglycemic medications may further exacerbate the metabolic derangements responsible for insulin resistance. Weight gain associated with the use of anti-hyperglycemic medications also may have detrimental effects on patient psychological well-being and quality of life [4], and can negatively influence compliance and persistence to anti-hyperglycemic medications [5,6]. Clearly, novel therapies to manage this disease are required.

Buckwheat has shown glucose-lowering potential in both humans and animal models [7-10]. Several mechanisms may be responsible for this effect, including the inhibition of enzymes responsible for breakdown of carbohydrates in the small intestine

that has been reported by others [9]. However, our observation of inhibited cellular glucose uptake after exposure of H4IIE cells to an extract prepared from common buckwheat may represent an alternative mechanism for reduced glycemia, especially if the inhibitory effect is present in the absorptive epithelial layer of the small intestine. To date, the inhibitory actions of buckwheat on cellular glucose uptake and transport in enterocytes have not been examined.

The mechanisms for inhibited cellular glucose uptake in cells treated with a concentrate prepared from common buckwheat remain unknown, although the insulin-mimetic properties of D-CI and MI are likely not involved. Experiments using chemical inhibitors have shown that phosphorylation of p42/44 ERK, resulting from exposure to the common buckwheat concentrate, is not associated with inhibition of glucose uptake. Although not related to glucose uptake inhibitory activity, the significance of p42/44 ERK activation for cell function has yet to be determined. More research is required to fully elucidate the effects of the common buckwheat extract on cell signaling pathways governing glucose uptake and metabolism. Gaining an understanding of how bioactive compounds present in buckwheat act at the cellular level to alter glucose metabolism is important for determining the suitability of buckwheat as an appropriate potential agent for altering glucose metabolism in T2DM.

Buckwheat is a source of numerous flavonoids, including rutin, orientin, vitexin, quercetin, isovitexin, quercetrin and isorientin [11], several of which inhibit facilitative glucose uptake by directly inhibiting activity of the GLUT2 glucose transporter isoform [12]. Other non-glycosylated polyphenols have been shown to inhibit glucose uptake under both sodium-free and sodium-dependent conditions [13], suggesting these compounds can inhibit active glucose transport in addition to facilitative glucose transport. Clearly, phytochemicals have the ability to directly inhibit glucose transporter activity. To date, direct inhibition of glucose transporters by compounds present in

buckwheat has not been investigated. Previous studies have utilized *Xenopus laevis* oocytes engineered to express single transporters as a model in which to assess direct inhibition of glucose transporters, mainly because expression of multiple glucose transporter isoforms in mammalian cell lines makes it difficult to identify inhibition of individual transporters. However, the Clone 9 liver cell line is reported in many publications as expressing only the GLUT1 facilitative glucose transporter isoform [14,15]. Therefore, this cell line may represent a mammalian model in which to study specific inhibition of GLUT1. Furthermore, introducing other glucose transporter isoforms followed by silencing of GLUT1 in this cell line could create a model for studying other individual glucose transporter isoforms in a mammalian system. However, GLUT3 expression has never been assessed in this cell line; therefore, these cells must first be screened for the presence of other glucose transporters before they can be used as a mammalian model for single transporter expression.

Ultimately, the goal of studying the glucose-lowering properties of buckwheat is to develop a food product to aid in glycemic management. Previous studies using baked products made with buckwheat flour have shown a glucose-lowering effect in individuals with T2DM after long-term consumption of the product [7,8]. Given that buckwheat has been shown to inhibit intestinal carbohydrases and has the potential to inhibit cellular glucose uptake in enterocytes, a reduction in the post-prandial glycemic response immediately after consuming a food product made with buckwheat would be a useful addition for improving glycemic control. However, no studies have determined the effects of consuming a food product made from buckwheat on the acute post-prandial glucose response. Furthermore, the effects of consuming a food product made from buckwheat on gastrointestinal incretin hormones, known to affect insulin secretion and glycemia, have not been determined. Thus, studies need to be conducted to test the feasibility of incorporating a reasonable amount of buckwheat into a food product for its

efficacy in reducing glycemia in humans. Additionally, the duration of buckwheat consumption required to observe beneficial effects on glycemia and other metabolic parameters has not been determined.

## 3.2 Hypotheses and Objectives

(1) Bioactive compound(s) in common buckwheat favourably modify acute glucose metabolism through a mechanism related to inhibition of glucose uptake in intestinal cells (Chapter 4 and Chapter 5).

Objectives:

- To determine if rutin and/or quercetin is the bioactive compound responsible for phosphorylation of p42/44 ERK and inhibition of glucose uptake observed upon treatment with the common buckwheat concentrate (Chapter 4)
- To determine if a solvent extract prepared from common buckwheat contains the bioactive compound(s) responsible for biological activities (phosphorylation of p42/44 ERK and inhibition of glucose uptake) previously reported with a buckwheat concentrate.
- To determine the effect of a buckwheat extract prepared from common buckwheat on glucose uptake in Caco2 cells.
- To determine the effect of a buckwheat extract prepared from common buckwheat on transepithelial glucose transport in polarized Caco2 cells.

(2) Inhibition of glucose uptake by buckwheat involves activation of pathways regulating glucose uptake and metabolism (Chapter 5 and Appendix 1).

Objectives:

- To determine if the glucose uptake inhibitory activity of buckwheat is retained in cells treated with inhibitors of several signaling pathways involved in glucose metabolism, including PI3Kγ, PKC, PKA, mammalian target of rapamycin (mTOR) and AMPK.
- To determine the effect of buckwheat on glucose production and the pathways affecting glucose production by measuring protein levels of key gluconeogenic enzymes (G6Pase and PEPCK) as well as activation of signaling pathways regulating these enzymes, including CREB.

(3) Bioactive compound(s) in buckwheat alter glucose metabolism by direct inhibition of glucose transporters (Chapter 6).

Objectives:

- To establish the Clone 9 liver cell line as a mammalian model in which to study inhibition of single glucose transporters, by first confirming sole expression of GLUT1 in this cell line.

(4) A food product made from whole grain common buckwheat flour reduces the acute post-prandial glucose response by raising levels of incretin hormones in individuals with T2DM (Chapter 7).

Objectives:

- To compare plasma glucose and insulin concentrations in both healthy participants and those with T2DM after consumption of a food product made from whole grain common buckwheat flour versus a food product made from rice flour.

- To compare plasma concentrations of the incretin hormones GIP and GLP-1, and other gastrointestinal satiety hormones, after consumption of a food product made from whole grain common buckwheat flour or rice flour in both healthy individuals and those with T2DM.

(5) Consumption of a food product made from whole grain common buckwheat flour for seven days favourably modifies fasting plasma glucose and lipids in individuals with T2DM (Chapter 7).

Objectives:

- To measure plasma glucose concentrations in both healthy individuals and those with T2DM after consumption of a food product made from whole grain common buckwheat flour or rice flour for seven days.
- To measure plasma triglycerides, total, LDL and HDL cholesterol, as well as apolipoprotein AI, B, and CIII in both healthy individuals and those with T2DM after consumption of a food product made from whole grain common buckwheat flour or rice flour for seven days.

## 3.3 Literature Cited

[1] ADVANCE Collaborative Group, Patel, A., MacMahon, S., Chalmers, J., Neal, B., Billot, L., Woodward, M., Marre, M., Cooper, M., Glasziou, P., Grobbee, D., Hamet, P., Harrap, S., Heller, S., Liu, L., Mancia, G., Mogensen, C.E., Pan, C., Poulter, N., Rodgers, A., Williams, B., Bompoint, S., de Galan, B.E., Joshi, R., and Travert, F. (2008). Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *N. Engl. J. Med.* 358, 2560-72.

[2] Duckworth, W., Abraira, C., Moritz, T., Reda, D., Emanuele, N., Reaven, P.D., Zieve, F.J., Marks, J., Davis, S.N., Hayward, R., Warren, S.R., Goldman, S., McCarren, M., Vitek, M.E., Henderson, W.G., Huang, G.D., and VADT Investigators. (2009). Glucose control and vascular complications in veterans with type 2 diabetes. *N. Engl. J. Med.* 360, 129-39.

[3] Hinnen, D., Nielsen, L.L., Waninger, A., and Kushner, P. (2006). Incretin mimetics and DPP-IV inhibitors: new paradigms for the treatment of type 2 diabetes. *J. Am. Board Fam. Med.* 19, 612-20.

[4] Clark, M. (2004). Is weight loss a realistic goal of treatment in type 2 diabetes? The implications of restraint theory. *Patient Educ. Couns.* 53, 277-83.

[5] Malone, M., Alger-Mayer, S.A., and Anderson, D.A. (2005). Medication associated with weight gain may influence outcome in a weight management program. *Ann. Pharmacother.* 39, 1204-8.

[6] Farmer, A., Kinmonth, A.L., and Sutton, S. (2006). Measuring beliefs about taking hypoglycaemic medication among people with Type 2 diabetes. *Diabet. Med.* 23, 265-70.

[7] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia and hyperlipidemia. In: *Proceedings of the 5th International Symposium on Buckwheat*, Lin, R., Zhou, M., Tao, Y., Li, J., Zhang, Z., Eds.; Agriculture Publishing House: Beijing, China, 458-64.

[8] Wang, J., Liu, Z., Fu, X., and Run, M. (1992). A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: *Proceedings of the 5th International Symposium on Buckwheat,* Lin, R., Zhou, M., Tao, Y., Li, J., Zhang, Z., Eds.; Agriculture Publishing House: Beijing, China, 465-7.

[9] Hosaka, T., Nii, Y., Tomotake, H., Ito, T., Tamanaha, A., Yamasaka, Y., Sasaga, S., Edazawa, K., Tsutsumi, R., Shuto, E., Okahisa, N., Iwata, S., and Sakai, T. (2011). Extracts of common buckwheat bran prevent sucrose digestion. *J. Nutr. Sci. Vitaminol. (Tokyo)* 57, 441-5.

[10] Kawa, J.M., Taylor, C.G., and Przybylski, R. (2003). Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. *J. Agric. Food Chem.* 51, 7287-91.

[11] Kreft, S., Knapp, M., and Kreft, I. (1999). Extraction of rutin from buckwheat (Fagopyrum esculentum Moench) seeds and determination by capillary electrophoresis. *J. Agric. Food Chem.* 47, 4649-52.

[12] Kwon, O., Eck, P., Chen, S., Corpe, C.P., Lee, J.H., Kruhlak, M., and Levine, M. (2007). Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J.* 21, 366-77.

[13] Johnston, K., Sharp, P., Clifford, M., and Morgan, L. (2005). Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett.* 579, 1653-7.

[14] Behrooz, A. and Ismail-Beigi, F. (1998). Induction of GLUT1 mRNA in response to azide and inhibition of protein synthesis. *Mol. Cell. Biochem.* 187, 33-40.

[15] Shetty, M., Loeb, J.N., Vikstrom, K., and Ismail-Beigi, F. (1993). Rapid activation of GLUT-1 glucose transporter following inhibition of oxidative phosphorylation in clone 9 cells. *J. Biol. Chem.* 268, 17225-32.

# Chapter 4: Biological Response of Hepatomas to an Extract of *Fagopyrum esculentum* M. (buckwheat) is Not Mediated by Inositols or Rutin

*Julianne M. Curran[1,], *Danielle M. Stringer[1,4], Brenda Wright[4], Carla G. Taylor[1,4], Roman Przybylski[3], Peter Zahradka[1,2,4]

Departments of [1]Human Nutritional Sciences and [2]Physiology, University of Manitoba, Winnipeg Manitoba, Canada

Department of [3]Chemistry and Biochemistry, University of Lethbridge, Lethbridge, Alberta, Canada

[4]The Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface Research Centre, Winnipeg, Manitoba, Canada

*These authors contributed equally to this work.

Reprinted with permission from: *Journal of Agricultural and Food Chemistry* 2010, 58: 3197-3204. 

## 4.1 Abstract

Buckwheat contains D-*chiro*-inositol (D-CI) and *myo*-inositol (MI), possible insulin-mimetic compounds; thus, we investigated the insulin-mimetic activities of a buckwheat concentrate (BWC), D-CI and MI on insulin signal transduction pathways and glucose uptake with H4IIE rat hepatoma cells. The BWC stimulated phosphorylation of p42/44 ERK and its downstream target, $p70^{S6K}$, on $Thr^{421}$. In contrast, D-CI, MI, rutin or its agylcone form, quercetin, did not activate these signal transduction proteins. Phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), another target of insulin, was also upregulated upon BWC treatment. We subsequently investigated the effects of the BWC on glucose uptake using H4IIE cells. Insulin and D-CI stimulated glucose uptake, while the BWC inhibited basal and insulin-stimulated glucose uptake. Although results from this work suggest that the BWC has insulin-mimetic effects on select protein phosphorylation events in H4IIE cells, D-CI and MI were not the active components responsible for the observed effects. The inhibition of glucose uptake by the BWC suggests that buckwheat may affect hepatic glucose metabolism, possibly by inhibiting glucose flux. Furthermore, the fact that D-CI and MI stimulated glucose uptake in H4IIE cells suggests that other compounds are responsible for inhibition of glucose uptake by the BWC.

## 4.2 Introduction

Insulin directly affects glucose uptake in skeletal muscle and adipose tissue through its regulation of the GLUT4 glucose transporter [1] whereas in the liver the effect is indirect. Although glucose entry into the hepatocyte is facilitated through the insulin–independent GLUT2 glucose transporter, binding of insulin to its receptor activates signal transduction pathways and enzymes involved in hepatic glucose metabolism, leading to increased glucose utilization through glycolysis, glycogenesis and suppressed gluconeogenesis [2]. Thus, initial increases in hepatocyte intracellular glucose concentrations are counteracted by activation of these pathways, eventually leading to a decrease in intracellular glucose concentrations. As a result, glucose enters the hepatocyte by facilitative diffusion through GLUT2, resulting in clearance of glucose from the portal vein and, consequently, a reduction in glycemia.

Buckwheat contains relatively high amounts of free D-*chiro*-inositol (D-CI), *myo*-inositol (MI) and galactosyl derivatives of D-CI known as fagopyritols [3]. Both D-CI and MI have been identified as components of inositol phosphoglycan (IPG) molecules that are released from cell membranes in response to insulin, and have demonstrated insulin-mimetic effects [4]. The insulin-like activities of isolated IPG and their chemically synthesized analogues have been widely investigated and are summarized elsewhere [5-7].

Like insulin, IPG such as D-CI have been shown to lower serum glucose levels in rats [8,9]. Among the reported insulin-mimetic effects of IPG are *in vitro* activation of enzymes and transcription of genes related to glucose metabolism, stimulation of glucose transport, GLUT4 translocation, glycogen synthesis, lipogenesis, and protein synthesis [5-7]. An IPG containing MI has been reported to modulate key intermediates in the insulin-signaling pathway, such as phosphatidylinositol 3-kinase (PI3K), extracellular related kinase (ERK) and glycogen synthase kinase-3 (GSK-3) [5-7].

Although it has been less studied, the D-CI-containing IPG also exerts insulin mimetic effects *in vitro* through the activation of key protein phosphatases known to be stimulated by insulin [5,7].

It has been previously demonstrated that intragastric administration of a buckwheat concentrate (BWC), containing D-CI, MI and fagopyritols, effectively lowered serum glucose concentrations in streptozotocin (STZ) rats in the fed state [10]. In humans, consumption of buckwheat is associated with a lower prevalence of hyperglycemia [11] and improved glucose tolerance in people with diabetes [12,13]. Although the antihyperglycemic effects of free D-CI and the BWC (containing D-CI) was presumed to be mediated via the insulin-mimetic activities of IPG, this was not directly investigated. Therefore, the purpose of the present study was to assess the insulin-mimetic activities of the BWC, D-CI and MI on activation of proteins in the insulin signaling pathway. Given the central role of the liver in glucose metabolism, the effects of BWC versus insulin on phosphorylation of signal transduction proteins and hepatic glucose uptake were investigated in H4IIE cells, a hepatoma cell line that has been previously used to characterize hepatic insulin signaling and glucose metabolism [14,15].

## 4.3 Materials and Methods

Tissue culture media, antibiotics, fetal bovine serum and Nunc tissue culture plates were purchased from Invitrogen. Insulin and MI were purchased from Sigma. D-CI was purchased from Industrial Research Limited. Polyclonal antibodies against phospho-Akt ($Ser^{473}$), phospho-GSK3 ($Ser^{21/9}$), phospho-Insulin receptor ($Tyr^{1146}$), p42/44 ERK, phospho-p42/44 ERK ($Thr^{202}/Tyr^{204}$), $p70^{S6K}$, phospho-$p70^{S6K}$ ($Thr^{389}$), phospho-$p70^{S6K}$ ($Thr^{421}$), p38 MAPK, phospho-38 MAPK ($Thr^{180}/Tyr^{182}$), phospho-STAT3

(Tyr$^{705}$) and phospho-S6 ribosomal protein (Ser$^{235/236}$) were purchased from Cell Signaling. The polyclonal antibody against phospho-Src (Tyr$^{529}$) was obtained from Biosource and phospho-insulin receptor substrate-I (Tyr$^{941}$) (IRS-1) was obtained from Santa Cruz. HRP-coupled anti-rabbit IgG was purchased from Bio-Rad.

Compounds used as selective inhibitors of signal transduction were obtained from Calbiochem (AG1024 and SB203580), New England Biolabs (PD98059), Biomol Inc. (LY294002 and PP1) and Sigma (Brefeldin A; 1-butanol). Inhibitor details are provided in Table 1. $^{3}$H-deoxyglucose was purchased from Perkin Elmer. General laboratory chemicals were purchased from Sigma and Fisher. Ultrapure chemicals (Tris, glycine, SDS, acrylamide, glycerol, Tween 20) were obtained from Invitrogen, Bio-Rad or Roche.

*Preparation of the BWC***.** The BWC was prepared from the Koto variety of buckwheat (*Fagopyrum esculentum*, Moench), which was provided by Kade Research (Morden, Manitoba, Canada). Details on preparation and analysis of the BWC have been previously described [10].

*Cell culture.* Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548) were cultured as previously described [16]. Briefly, cells were maintained in $\alpha$-modified Eagle's media containing 10% fetal bovine serum, 2 mM glutamine, 25 μg$\cdot$mL$^{-1}$ streptomycin and 25 units$\cdot$mL$^{-1}$ penicillin. Cells were plated and grown to 70% confluence. All cells were placed into serum-free medium for 72 h before addition of stimulating agents to ensure entry into a quiescent state.

*Western blotting.* Cultures of quiescent H4IIE cells, in 12-well culture dishes containing 2 mL serum-free medium, were stimulated by direct addition of the indicated compounds (volumes of additions were 10 μL or less) without replacing the medium. Insulin was dissolved in water and added directly to cells at a concentration of 250 nM unless otherwise indicated. Two μL (0.1% v/v final concentration) of the BWC were added

directly to cells unless otherwise indicated. This amount was chosen on the basis of equivalence to insulin with respect to p42/44 ERK phosphorylation. MI and D-CI were dissolved in water at an equal concentration to the amounts present in the BWC (*10*) and 2 μL of each solution was added directly to cells. The final concentrations of D-CI and MI were $5.72 \times 10^{-10}$ M and $5.56 \times 10^{-10}$ M, respectively. Based on initial time course experiments (data not shown), it was determined that exposure to treatments for 6 minutes was sufficient to elicit a response for phosphorylation of p42/44 ERK; therefore 6 minutes was chosen for all phosphorylation experiments, in agreement with previous observations [17].

Inhibitors were added 10 minutes before the stimulating agents. The cells in 12-well culture dishes were incubated with stimulating agents for 6 minutes and rinsed with PBS. Cellular protein extract preparation and Western blotting were carried out as previously described [18], with the exception that all incubations were carried out at room temperature.

*Assay of $^{3}$H-deoxyglucose uptake in H4IIE cells.* $^{3}$H-deoxyglucose uptake was assayed as previously described [19]. Inhibitors were added for 10 minutes prior to the 20 minute stimulation with treatments (insulin, $10^{-6}$ M; BWC, D-CI and MI, 0.4% v/v unless otherwise indicated).

*Statistical Analyses***.** All analyses were performed using SAS statistical software (SAS v.9.1, SAS Institute Inc., Cary, NC). Statistical significance between treatment groups for Western blot data was determined using a mixed model analysis with a random intercept for the repetitions and by estimate statements for individual comparisons of treatments versus the control. Statistical significance between treatments for glucose uptake data was determined by one-way ANOVA and by Duncan's multiple range test for post-hoc means testing. Differences were accepted as significant at $p<0.05$. Data are presented

as means ± SEM (n=3) with the exception of Figure 4, which represents one-time experiments.

## 4.4 Results

*Effects of the buckwheat concentrate on protein phosphorylation.* To investigate the mechanism by which the BWC lowers serum glucose [10], we monitored the phosphorylation status of key intracellular modulators of insulin signaling after treatment with BWC, insulin, D-CI and MI. As shown in Figure 4.1, neither BWC, D-CI nor MI stimulated changes in insulin receptor, IRS-1, Akt, Src, STAT3 and GSK3 phosphorylation, although insulin elicited the expected responses. In contrast, the phosphorylation of p42/44 ERK and p38 MAPK was increased by both insulin and the BWC, while neither D-CI nor MI had any effect (Figure 4.2A and 4.2B). These results were confirmed in FAO hepatoma cells (data not shown). Finally, to establish if p42/44 ERK activation by insulin and the BWC is mediated by a common signal transduction pathway, we compared the effect of combining these agents versus the agents alone. Our results indicate that the actions of insulin and the BWC on p42/44 ERK, but not p38 MAPK, are additive (Figure 4.2C and 4.2D).

*Cell signaling pathways activated by the BWC***.** The intracellular signaling pathways that contribute to the actions of the BWC were evaluated through the use of selective inhibitors of phospholipase D (PLD), Src kinase (Src), and Arf3 in conjunction with Western blot analysis of protein phosphorylation. Figures 4.3A and 4.3B show representative results for insulin receptor and p42/44 ERK phosphorylation by insulin and the BWC when PLD, Src, and Arf3 were inhibited. Phosphorylation of the insulin receptor was stimulated by insulin only, and PLD, Src, and Arf3 were not required. As seen previously (Figure 4.1A), the BWC did not stimulate phosphorylation of the insulin receptor. However, as indicated in Figure 4.3B, the insulin and BWC stimulation of

p42/44 ERK phosphorylation was blocked by 1-butanol, a PLD inhibitor. In contrast, the Src kinase inhibitor PP1 affected only the stimulation of p42/44 ERK by the BWC. Arf3, which is sensitive to brefeldin, is not involved in this process.

We also compared the effects of the BWC versus insulin on phosphorylation of proteins downstream from p42/44 ERK. As shown in Figure 4.4A, the BWC stimulated the phosphorylation of $p70^{S6K}$ ($Thr^{421}$), but not $p70^{S6K}$ ($Thr^{389}$). In contrast, insulin stimulates the phosphorylation of $p70^{S6K}$ on both residues. Phosphorylation of ribosomal protein S6, which is immediately downstream from $p70^{S6K}$, occurred in the presence of insulin but not the BWC. The latter result is not surprising since S6 phosphorylation requires fully active $p70^{S6K}$, which is achieved when it is phosphorylated on both $Thr^{421}$ and $Thr^{389}$ [20]. Figure 4.4B and 4.4C show that stimulation of $p70^{S6K}$ ($Thr^{421}$) by both insulin and the BWC is p42/44 ERK dependent (PD98059 sensitive), but is independent of PI3-kinase (LY294002 sensitive). In contrast, insulin-dependent stimulation of $p70^{S6K}$ ($Thr^{389}$) phosphorylation requires activation of PI3K but is independent of p42/44 ERK (Figure 4.4c). These results illustrate that the BWC is not capable of activating PI3-kinase, which is a critical mediator of insulin signaling [21].

*Interactions between p42/44 ERK and p38 MAPK.* To establish whether the BWC operates through a common pathway to activate p42/44 ERK and p38 MAPK, selective inhibitors of these signaling enzymes were employed. As expected, PD98059, a potent inhibitor of p42/44 ERK phosphorylation [18] blocked activation of p42/44 ERK in response to the BWC (Figure 4.5A). Likewise, SB203580, a p38 MAPK inhibitor [22] blocked the increase in phosphorylation of p38 MAPK caused by the BWC (Figure 4.5B). Interestingly, PD98059 also prevented the stimulation of p38 MAPK (Figure 5.5B), while SB203580 inhibited p42/44 ERK phosphorylation (Figure 4.5A), suggesting crosstalk between the pathways.

In addition, we observed that SB203580 did not interfere with BWC- and insulin-dependent phosphorylation of $p70^{S6K}$ on $Thr^{421}$, but rather increased it (Figure 4.6A). This contrasts with the previous result wherein PD98059 clearly prevented this event (Figure 4.4B and 4.4C), thus implying p38 MAPK operates as a repressor while p42/44 ERK enhances phosphorylation of $p70^{S6K}$ on $Thr^{421}$ by BWC and insulin. Furthermore, inhibition of p38 MAPK promoted insulin-stimulated phosphorylation of $p70^{S6K}$ on $Thr^{389}$ (Figure 4.4D). These results suggest p42/44 ERK and p38 MAPK mediate distinct signal transduction pathways activated by the BWC and insulin.

*Glucose uptake in H4IIE cells is stimulated by insulin, but inhibited by the BWC.* In addition to our examination of signal transduction pathways, we compared the ability of the BWC, insulin, D-CI and MI to stimulate glucose uptake by H4IIE hepatoma cells. As shown in Figure 4.7A, insulin and D-CI stimulate glucose uptake above basal levels. While MI also appears to slightly increase glucose uptake, the data were not statistically different from untreated cells. In contrast, the BWC inhibited basal glucose uptake. We investigated the dose-dependent effects of the BWC on inhibition of basal glucose uptake and found that 0.5 μL of the BWC (0.1% v/v final concentration) had no effect on basal glucose uptake whereas 2 μL and 10 μL of the BWC (0.4% and 2%, respectively) apparently blocked basal glucose uptake (Figure 6.7B). Interestingly, addition of the BWC to cells 10 minutes prior to treatment with insulin also prevented glucose uptake (Figure 4.7C).

In addition to D-CI and MI, buckwheat contains an abundant amount of rutin. Both rutin and the aglycone form of rutin, quercetin, have been previously shown to inhibit glucose uptake [23]. We therefore examined the effect of rutin and quercetin relative to the BWC on glucose uptake by H4IIE cells. Rutin had no effect on glucose uptake, but 50 μM quercetin was equipotent with the BWC (Figure 4.7D). Neither rutin

nor quercetin stimulated phosphorylation of 42/44 ERK (Figure 4.7E and 4.7F). Since ethanol/water, which was used to prepare the BWC, would not be expected to remove rutin and quercetin from the buckwheat based on incompatible polarities, we analyzed the BWC for rutin and quercetin. Neither compound was detected (data not shown). We therefore conclude that rutin and/or quercetin are not responsible for the glucose uptake inhibitory actions of the BWC.

This study also examined the contribution of various signal transduction mediators to inhibition of glucose uptake by the BWC. Unfortunately, identifying the contribution of p42/44 ERK to this process was not possible, as PD98059 was a potent inhibitor of insulin-mediated uptake (Figure 4.8A). As expected, insulin operated through the insulin receptor, as indicated by the decrease in glucose uptake seen with the insulin receptor tyrosine kinase inhibitor AG1024. On the other hand, addition of 1-butanol, a PLD inhibitor, had a partial effect, since uptake was reduced only to basal levels. When the same inhibitors were tested with the BWC, no changes were observed (Figure 4.8B). These results suggest the actions of the BWC on glucose uptake are independent of p42/44 ERK. We performed a separate series of experiments with the p38 MAPK inhibitor SB203580. This compound was unable to affect the actions of either insulin or the BWC on glucose uptake (Figure 4.8C). These results confirm that p42/44 ERK and p38 MAPK operate via distinct pathways for both of these agents, with p42/44 ERK being linked to glucose uptake while p38 MAPK is not.

**Table 4.1 Inhibitors of signal transduction proteins in the insulin signaling pathway.**

| Compound | Inhibits phosphorylation of | Working Concentration | Reference |
|---|---|---|---|
| PD98059 | P42/44 ERK | $10^{-5}$ M | Yau et al., 1999 |
| Brefeldin A | phospholipases | 180 µM | Li et al., 1998 |
| 1-butanol | PLD1 | 0.3% | Morton et al., 1995 |
| PP1 | Src | $10^{-5}$ M | Zahradka et al, 2004 |
| AG1024 | Insulin receptor | 0.5 x $10^{-5}$ M | Zahradka et al, 2004 |
| LY294002 | PI3K | $10^{-5}$ M | Saward & Zahradka, 1997 |
| SB203580 | P38 MAPK targets | $10^{-5}$ M | Cuenda & Rousseau, 2007 |

Inhibitors were prepared in dimethyl sulfoxide (DMSO) unless otherwise indicated.

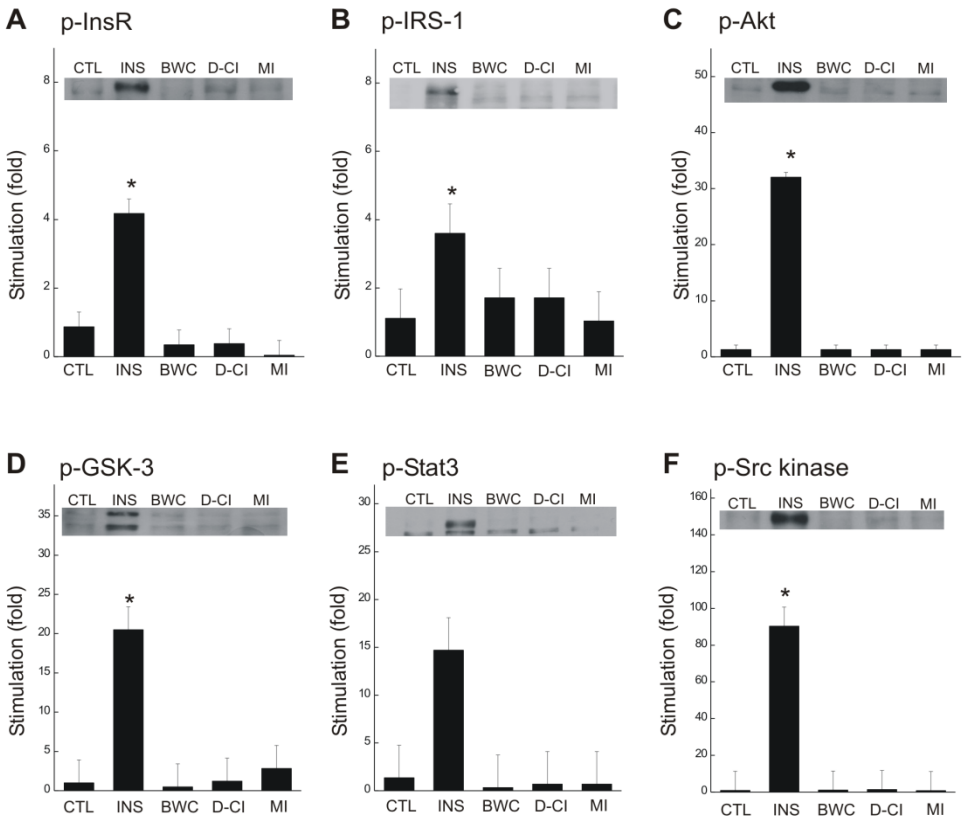


**Figure 4.1 Comparative actions of insulin, buckwheat concentrate, D-CI and MI on protein phosphorylation in H4IIE cells.** Cells were treated with insulin (INS; 250 nM), buckwheat concentrate (BWC; 0.1% v/v), D-chiro inositol (D-CI; 5.72 x $10^{-10}$ M) and myo-inositol (MI; 5.56 x $10^{-10}$ M) individually for 6 minutes, with untreated cells serving as the control (CTL). Protein phosphorylation was monitored by Western blotting with phospho-specific antibodies for (A) the insulin receptor, (B) IRS-1, (C) Akt, (D) GSK-3, (E) Stat3 and (F) Src kinase. Representative blots are shown in the insets. Band intensities were quantified by scanning densitometry and are plotted as means ± SEM (n=3) relative to control (set to 1). Significant differences ($p<0.05$) between treatments versus the control (*) are indicated. The p-value for the comparison of insulin-stimulated phosphorylation of Stat3 versus the control was p=0.06.

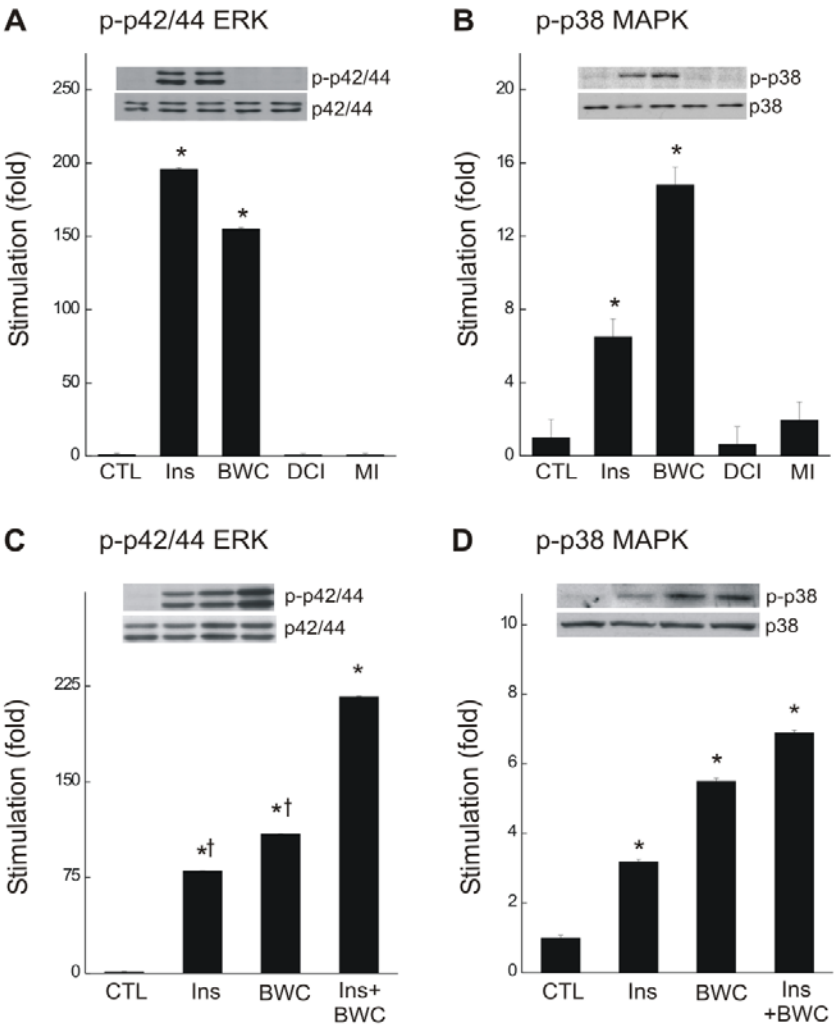


**Figure 4.2 Activation of p42/44 ERK and p38 MAPK by insulin and the buckwheat concentrate in H4IIE cells.** Cells were treated with insulin (Ins; 250 nM), the buckwheat concentrate (BWC; 0.1% v/v), D-chiro inositol (D-CI; 5.72 x $10^{-10}$ M) or myo-inositol (MI; 5.56 x $10^{-10}$ M) for 6 minutes, with untreated cells serving as the control (CTL). Phosphorylation was monitored by Western blotting with phospho-specific antibodies for A) and C) p42/44 ERK and B) and D) p38 MAPK. Representative blots are shown in the insets. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Significant differences ($p<0.05$) between treatments versus the control (*) are indicated. $^{t}$ denotes significant difference compared to Ins+BWC ($p<0.05$).

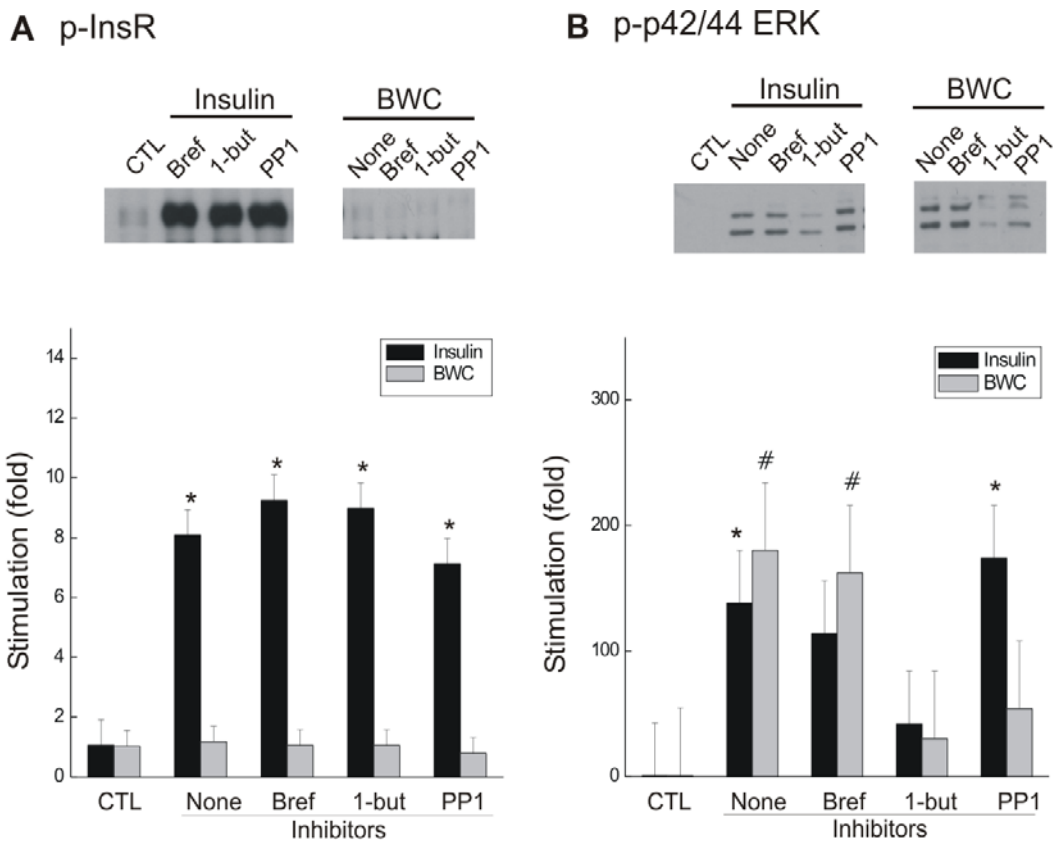


**Figure 4.3 Insulin- and buckwheat concentrate-stimulated phosphorylation of the insulin receptor and p42-44 ERK after inhibition of phospholipase D, Src kinase and Arf3 in H4IIE cells.** Following a 15 minute pre-incubation with butanol (0.3 % v/v; inihibits phospholipase D), PP1 ($10^{-5}$ M; inhibits Src kinase), or brefeldin ($1.8 \times 10^{-4}$ M; inhibits Arf), cells were treated with either insulin (250 nM) or the buckwheat concentrate (BWC; 0.1% v/v) for 6 minutes. The effects of inhibitors on BWC and insulin-stimulated phosphorylation of A) insulin receptor (InsR) and B) p42/44 ERK are shown. Representative blots are presented above each graph. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). An * indicates statistical significance ($p<0.05$) for insulin ± inhibitors versus the control and # indicates statistical significance ($p<0.05$) for BWC ± inhibitors versus the control.

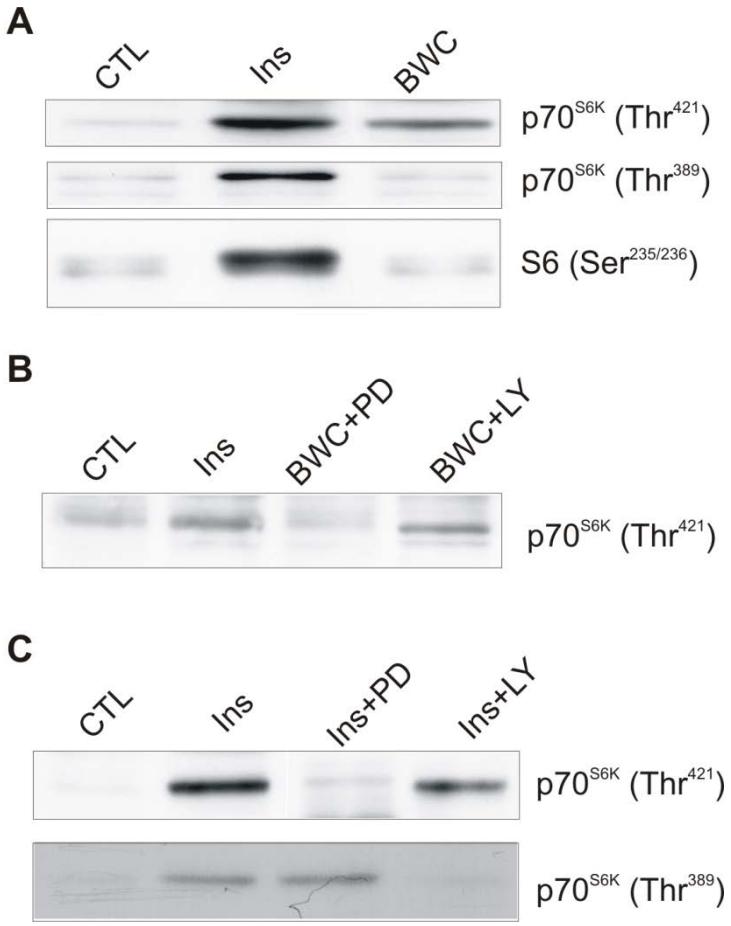


**Figure 4.4 Contrasting effects of insulin and the buckwheat concentrate on activation of p70$^{S6K}$ and ribosomal protein S6 in H4IIE cells.** (A) Cells were treated with insulin (INS; 250 nM) or the buckwheat concentrate (BWC; 0.1% v/v) for 6 minutes, with untreated cells serving as the control (CTL). (B) Cells were pre-treated with PD98059 (PD; $10^{-5}$ M; inhibits p42/44 ERK) or LY294002 (LY; $10^{-5}$ M; inhibits PI3K) for 15 minutes prior to treatment with the BWC. (C) Cells were pretreated PD ($10^{-5}$ M) or LY ($10^{-5}$ M) for 15 minutes prior to treatment with insulin (Ins; 250 nM). Phosphorylation of p70$^{S6K}$ (Thr$^{421}$), p70$^{S6K}$ (Thr$^{389}$) and ribosomal protein S6 (Ser$^{235/236}$) were assessed using Western blotting. Representative blots (n=1) are shown.

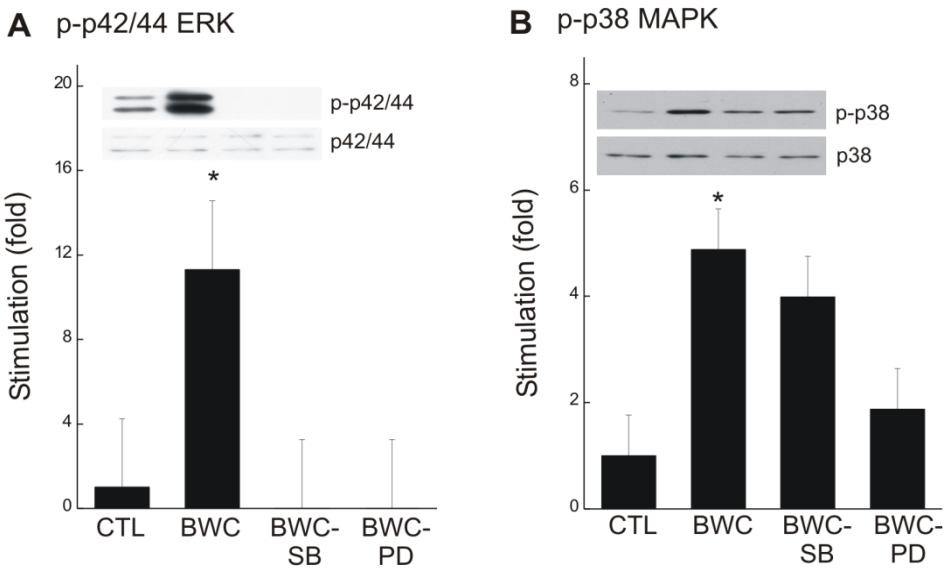


**Figure 4.5 Mutual regulation of buckwheat concentrate-stimulated p42/44 ERK and p38 MAPK phosphorylation in H4IIE cells.** Following a 15 minute pre-incubation with $10^{-5}$ M of the indicated inhibitors, H4IIE cells were treated with the buckwheat concentrate (BWC; 0.1% v/v) for 6 minutes, with untreated cells serving as the control (CTL). Band intensities of A) p42/44 ERK and B) p38 MAPK on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Significant differences ($p<0.05$) between treatments versus the control (*) are indicated.

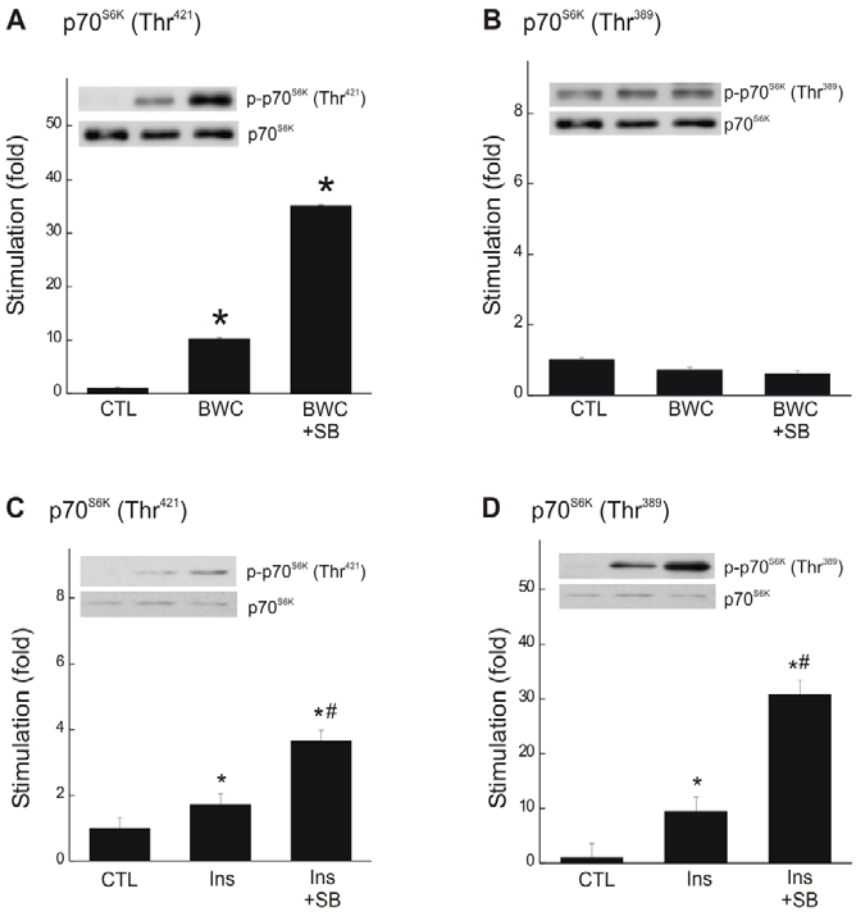


**Figure 4.6 Insulin- and buckwheat-stimulated phosphorylation of p70$^{S6K}$ in response to p38 MAPK inhibition in H4IIE cells.** Following a 15 minute preincubation with the p38 MAPK inhibitor SB203580 (SB; $10^{-5}$ M), H4IIE cells were treated with Ins (250 nM) or the buckwheat concentrate (BWC; 0.1% v/v) for 10 minutes, with untreated cells serving as the control (CTL), and blotted for phospho-p70$^{S6K}$ (Thr$^{421}$) (A and C) or p70$^{S6K}$ (Thr$^{389}$) (B and D). Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SEM (n=3). Significant differences ($p<0.05$) between treatments versus the control are indicated (*). Significant differences ($p<0.05$) between Ins+SB203580 versus the control are indicated ($^{\#}$).

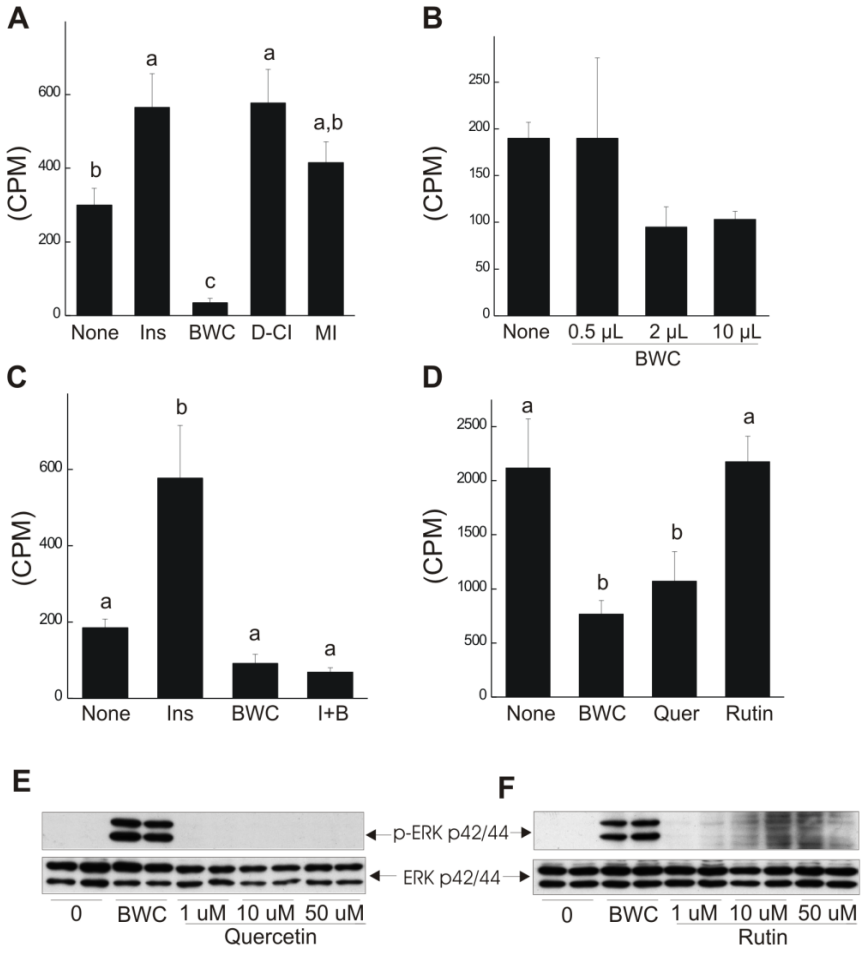


**Figure 4.7 The buckwheat concentrate inhibits $^{3}$H deoxyglucose uptake in H4IIE cells.** A) The effects of insulin (INS; $10^{-6}$ M), the buckwheat concentrate (BWC; 0.4% v/v), D-chiro inositol (0.4% v/v) and myo-inositol (0.4% v/v) on glucose uptake; B) the dose-dependent effects of the BWC on inhibition of glucose uptake; C) the effect of the BWC (0.4% v/v) on basal and insulin (250 nM)-stimulated glucose uptake; D) The effects of quercetin (Quer; 50 µM) and rutin (50 µM) on glucose uptake; E) dose-dependent effects of quercetin on phosphorylation of p42/44 ERK; F) dose-dependent effects of rutin on phosphorylation of p42/44 ERK. The data are presented as means ± SEM (n=3). Statistical significance ($p<0.05$) was determined by one-way ANOVA and Duncan's multiple range test. Bars with different letters are significantly different.

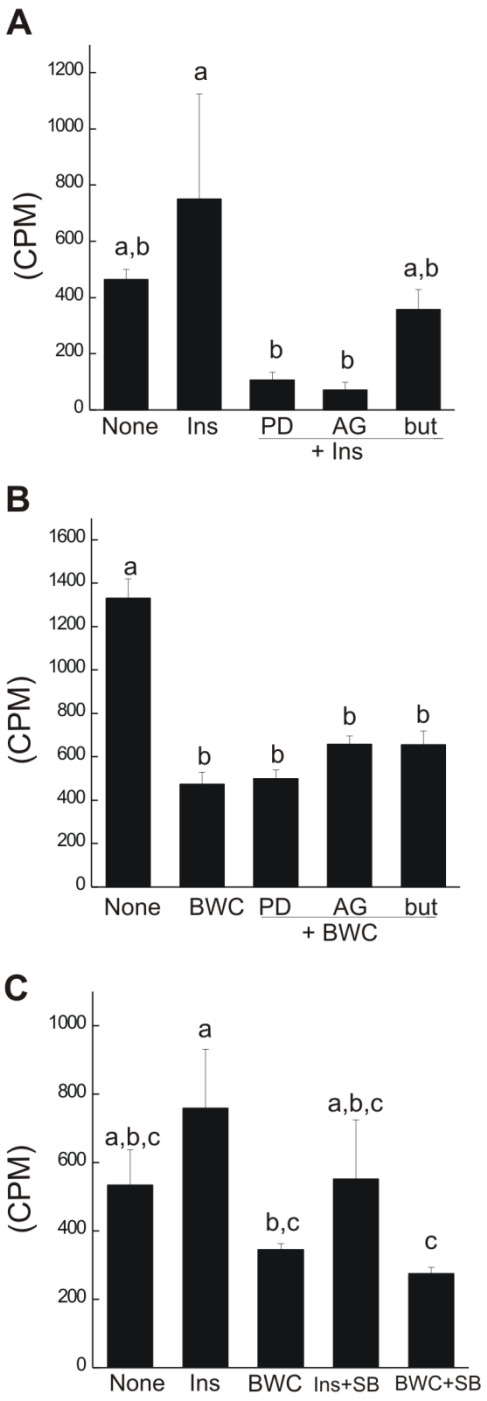


**Figure 4.8 Inhibiting p42/44 ERK, the insulin receptor, phospholipase D, and p38 MAPK ablates insulin-stimulated glucose uptake but has no effect on the inhibition of glucose uptake by the buckwheat concentrate in H4IIE cells.** H4IIE cells were treated with PD98059 (PD; $10^{-5}$ M), AG1024 (AG; $10^{-5}$ M), or 1-butanol (but; 0.3% v/v) 10 minutes before addition of A) insulin (Ins; 250 nM) or B) the buckwheat concentrate (BWC; 0.4% v/v). C) H4IIE cells were treated with SB203580 ($10^{-5}$ M) 10 minutes before addition of insulin (Ins) or BWC (0.4% v/v). Data are presented as means ± SEM (n=3). Statistical significance ($p<0.05$) was determined by one-way ANOVA and Duncan's multiple range test. Bars with different letters are significantly different.

## 4.5 Discussion

Oral administration of a BWC significantly lowers blood glucose levels in STZ rats [10]. Since the BWC contains the insulin-mimetic compounds D-CI and MI [3], it was hypothesized that the BWC would have insulin mimetic effects on cell signal transduction proteins and would promote glucose uptake in H4IIE rat hepatoma cells. The results of the present study indicate that the BWC does not function as an insulin mimetic since it does not activate the same pathways as insulin. Likewise, neither D-CI nor MI stimulated phosphorylation of the proteins examined in this study. In contrast, both insulin and the BWC activated p42/44 ERK and p38 MAPK. Furthermore, it was expected that the presence of D-CI and MI in the BWC would enhance glucose uptake; however, BWC inhibited basal glucose uptake in the H4IIE cells, contrary to the effects of insulin, D-CI and MI. This suggests that D-CI and MI may not be responsible for the previously observed antihyperglycemic effects of the BWC.

Compounds having insulin mimetic properties show promise in the treatment of diabetes [24]. In addition to pharmacological insulin mimetics [25], naturally-derived insulin mimetics also exist, including D-CI and MI-containing IPG [5-7]. The insulin-mimetic effects of free D-CI and MI have not been evaluated previously at the cell signal transduction level; however, free D-CI has demonstrated blood glucose lowering effects in animal models of diabetes presumably through a mechanism related to insulin-like activities of IPG [8,9]. At the same time, in the current study, both isolated D-CI and MI did not activate intermediates in the insulin signaling pathway, suggesting that these compounds do not act as insulin mimetics. Furthermore, the BWC, which contained D-CI and MI, did not activate insulin signaling intermediates, suggesting that the presence of these compounds is not responsible for previously observed anti-hyperglycemic effects of the BWC [10]. Buckwheat contains a number of polyphenolic compounds including fagopyritols, flavonoids (rutin, quercetin, catechins, epicatechin, hyperoside and

proanthocyanidins), and lignans, and although we have ruled out rutin and quercetin, it is possible that one or more of these compounds is responsible for the insulin mimetic effects of the BWC as several bioflavonoid and phenol compounds have been reported to improve hyperglycemia in diabetes by affecting glucose transport and insulin-receptor function [26]. It should be noted, however, that many polyphenolic compounds are metabolized in the intestine and do not circulate in their native forms in the bloodstream, but rather as metabolites. For example, quercetin can be rapidly conjugated to glucuronic acid or sulfate during first pass metabolism, and can also be subject to methylation [27]. Therefore, to provide a more complete representation of *in vivo* conditions, future *in vitro* studies should test the effects of such polyphenol metabolites. Nonetheless, unconjugated quercetin (but not rutin) has been detected in plasma from humans who have consumed 100 mg of rutin, suggesting that free quercetin, derived from deconjugation of rutin, does circulate in plasma and may elicit biological effects [28].

Treatment of H4IIE cells with insulin and the BWC stimulated phosphorylation of p42/44 ERK and p38 MAPK (Figure 4.2A and 4.2B). As a result, treatment with the BWC also stimulated phosphorylation of p42/44 ERK-dependent p70S6K$^{(Thr421)}$, but not PI3K-dependent p70S6K$^{(Thr389)}$; consequently, the BWC did not activate ribosomal S6, which requires phosphorylation of both Thr$^{241}$ and Thr$^{389}$ of p70S6K. Interestingly, both insulin and the BWC require PLD activation for phosphorylation of p42/44 ERK; however, the BWC also requires phosphorylation of Src kinase, suggesting the BWC phosphorylates p42/44 ERK by activating a pathway that is separate from insulin. This is further supported by the observed additive effects of insulin and the BWC on p42/44 ERK phosphorylation (Figure 4.2C).

Perhaps the most surprising result from the current study is the inhibition of both basal and insulin-stimulated glucose uptake by the BWC (Figure 4.8A). Although both

insulin and the BWC stimulate phosphorylation of p42/44 ERK, their respective effects on glucose uptake are opposite and, furthermore, glucose uptake by insulin is dependent on p42/44 ERK, while there appears to be no role of p42/44 ERK in inhibition of glucose uptake by the BWC (Figures 4.8E and 4.8F). This latter point reveals that activation of p42/44 ERK and inhibition of glucose uptake by the BWC are two unrelated, independent processes and highlights a divergent pathway of p42/44 ERK signaling. Inhibition of PLD had no effect on the inhibition of glucose uptake by the BWC.

Overall, the anti-hyperglycemic effects of buckwheat are not due to the insulin mimetic activities of naturally present inositol phosphoglycans or rutin and its aglycone directly on the liver, although the effect of the BWC in hepatocytes could somehow be related to its antihyperglycemic actions elsewhere in the body. A similar phenomenon of inhibited glucose uptake in the small intestine could potentially explain reduced hyperglycemia observed in rats; if so, buckwheat may represent a useful dietary agent for management of hyperglycemia. The mechanism of inhibited basal and insulin stimulated glucose uptake by the BWC in H4IIE cells can be addressed in future studies. Further research can clarify the significance of these findings in terms of glucose metabolism and can also elucidate the active component in the BWC responsible for the observed effects.

## 4.6 Literature Cited

[1] Kanzaki, M. and Pessin, J.E. (2001). Signal integration and the specificity of insulin action. *Cell Biochem. Biophys.* 35, 191-209.

[2] Wahren, J. and Ekberg, K. (2007). Splanchnic regulation of glucose production. *Annu. Rev. Nutr.* 27, 329-45.

[3] Horbowicz, M. and Obendorf, R.L. (1994). Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. *Seed Sci. Res.* 4, 385-405.

[4] Larner, J., Huang, L.C., Schwartz, C.F., Oswald, A.S., Shen, T.Y., Kinter, M., Tang, G.Z., and Zeller, K. (1988). Rat liver insulin mediator which stimulates pyruvate dehydrogenase phosphate contains galactosamine and D-chiroinositol. *Biochem. Biophys. Res. Commun.* 151, 1416-26.

[5] Field, M.C. (1997). Is there evidence for phospho-oligosaccharides as insulin mediators? *Glycobiology* 7, 161-8.

[6] Jones, D.R. and Varela-Nieto, I. (1998). The role of glycosyl-phosphatidylinositol in signal transduction. *Int. J. Biochem. Cell Biol.* 30, 313-26.

[7] Jones, D.R. and Varela-Nieto, I. (1999). Diabetes and the role of inositol-containing lipids in insulin signaling. *Mol. Med.* 5, 505-14.

[8] Fonteles, M.C., Almeida, M.Q., and Larner, J. (2000). Antihyperglycemic effects of 3-O-methyl-D-chiro-inositol and D-chiro-inositol associated with manganese in streptozotocin diabetic rats. *Horm. Metab. Res.* 32, 129-32.

[9] Ortmeyer, H.K., Bodkin, N.L., Lilley, K., Larner, J., and Hansen, B.C. (1993). Chiroinositol deficiency and insulin resistance. I. Urinary excretion rate of chiroinositol is directly associated with insulin resistance in spontaneously diabetic rhesus monkeys. *Endocrinology* 132, 640-5.

[10] Kawa, J.M., Taylor, C.G., and Przybylski, R. (2003). Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. *J. Agric. Food Chem.* 51, 7287-91.

[11] Zhang, H.W., Zhang, Y.H., Lu, M.J., Tong, W.J., and Cao, G.W. (2007). Comparison of hypertension, dyslipidaemia and hyperglycaemia between buckwheat seed-consuming and non-consuming Mongolian-Chinese populations in Inner Mongolia, China. *Clin. Exp. Pharmacol. Physiol.* 34, 838-44.

[12] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia and hyperlipidemia. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, pp 458-64.

[13] Wang J, Liu Z, Fu X, Run M (1992) A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, 465-7.

[14] Dickens, M., Svitek, C.A., Culbert, A.A., O'Brien, R.M., and Tavare, J.M. (1998). Central role for phosphatidylinositide 3-kinase in the repression of glucose-6-phosphatase gene transcription by insulin. *J. Biol. Chem.* 273, 20144-9.

[15] Sharfi, H. and Eldar-Finkelman, H. (2008). Sequential phosphorylation of insulin receptor substrate-2 by glycogen synthase kinase-3 and c-Jun NH2-terminal kinase plays a role in hepatic insulin signaling. *Am. J. Physiol. Endocrinol. Metab.* 294, E307-15.

[16] Yau, L., Elliot, T., Lalonde, C., and Zahradka, P. (1998). Repression of phosphoenolpyruvate carboxykinase gene activity by insulin is blocked by 3-aminobenzamide but not by PD128763, a selective inhibitor of poly(ADP-ribose) polymerase. *Eur. J. Biochem.* 253, 91-100.

[17] Yau, L. and Zahradka, P. (1997). Immunodetection of activated mitogen-activated protein kinase in vascular tissues. *Mol. Cell. Biochem.* 172, 59-66.

[18] Yau, L., Lukes, H., McDiarmid, H., Werner, J., and Zahradka, P. (1999). Insulin-like growth factor-I (IGF-I)-dependent activation of p42/44 mitogen-activated protein kinase occurs independently of IGF-I receptor kinase activation and IRS-1 tyrosine phosphorylation. *Eur. J. Biochem.* 266, 1147-57.

[19] Harrison, S.A., Buxton, J.M., Helgerson, A.L., MacDonald, R.G., Chlapowski, F.J., Carruthers, A., and Czech, M.P. (1990). Insulin action on activity and cell surface disposition of human HepG2 glucose transporters expressed in Chinese hamster ovary cells. *J. Biol. Chem.* 265, 5793-801.

[20] Hou, Z., He, L., and Qi, R.Z. (2007). Regulation of s6 kinase 1 activation by phosphorylation at ser-411. *J. Biol. Chem.* 282, 6922-8.

[21] Asano, T., Fujishiro, M., Kushiyama, A., Nakatsu, Y., Yoneda, M., Kamata, H., and Sakoda, H. (2007). Role of phosphatidylinositol 3-kinase activation on insulin action and its alteration in diabetic conditions. *Biol. Pharm. Bull.* 30, 1610-6.

[22] Cuenda, A. and Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim. Biophys. Acta* 1773, 1358-75.

[23] Johnston, K., Sharp, P., Clifford, M., and Morgan, L. (2005). Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett.* 579, 1653-7.

[24] Srivastava, A.K. and Mehdi, M.Z. (2005). Insulino-mimetic and anti-diabetic effects of vanadium compounds. *Diabet. Med.* 22, 2-13.

[25] Air, E.L., Strowski, M.Z., Benoit, S.C., Conarello, S.L., Salituro, G.M., Guan, X.M., Liu, K., Woods, S.C., and Zhang, B.B. (2002). Small molecule insulin mimetics reduce food intake and body weight and prevent development of obesity. *Nat. Med.* 8, 179-83.

[26] Jung, U.J., Lee, M.K., Jeong, K.S., and Choi, M.S. (2004). The hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice. *J. Nutr.* 134, 2499-503.

[27] Day, A.J., Mellon, F., Barron, D., Sarrazin, G., Morgan, M.R., and Williamson, G. (2001). Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. *Free Radic. Res.* 35, 941-52.

[28] Erlund, I., Kosonen, T., Alfthan, G., Maenpaa, J., Perttunen, K., Kenraali, J., Parantainen, J., and Aro, A. (2000). Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur. J. Clin. Pharmacol.* 56, 545-53.

# Chapter 5: Inhibition of facilitative glucose uptake by common buckwheat contributes to reduced glucose transport

Danielle M. Stringer[1,3], Carla G. Taylor[1,2,3], Peter Zahradka[1,2,3]

Departments of [1]Human Nutritional Sciences and [2]Physiology, University of Manitoba, Winnipeg Manitoba, Canada

[3]The Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface Research Centre, Winnipeg, Manitoba, Canada

## 5.1 Abstract

Common buckwheat (variety Koto) has previously demonstrated glucose-lowering effects in both healthy rats and those with type 1 diabetes. Although the mechanism has not been fully characterized, reported inhibition of cellular glucose uptake may contribute to reduced glycemia if present in the small intestine. The main purpose of the current study was to determine how compounds in common buckwheat inhibit intestinal glucose uptake and to explore the signaling pathways involved. 2-deoxy-D-glucose uptake was significantly reduced in Caco2 cells treated with an extract prepared from common buckwheat (BWE), suggesting buckwheat has the ability to inhibit intestinal glucose uptake. This inhibition resulted in significantly lower transepithelial glucose transport across polarized Caco2 monolayers. HPLC fractionation of the BWE revealed that treatment with only one of these fractions was associated with inhibited glucose uptake. Interestingly, glucose production in H4IIE cells was elevated by treatment with the buckwheat extract, and was accompanied by higher levels of the gluconeogenic enzymes glucose 6-phosphatase and phosphoenolpyruvate carboxykinase. Buckwheat treatment stimulated phosphorylation of CREB via a mechanism independent of cyclic AMP production and PKA, the typical pathway of CREB phosphorylation. Although phosphorylation of MSK-1, another CREB kinase was stimulated by buckwheat, MSK-1 gene silencing experiments suggest MSK-1 is not the kinase responsible for stimulation of CREB phosphorylation by buckwheat. Further experiments are required to fully characterize the pathway of CREB phosphorylation by buckwheat.

## 5.2 Introduction

Buckwheat is a broad-leafed herbaceous annual plant that is often classified and treated as a cereal crop. Epidemiological evidence from China has shown a significantly lower prevalence of hyperglycemia in a community consuming buckwheat as a staple food compared to a community that subsisted primarily on rice, suggesting a potential role for buckwheat in glycemic control [1]. Similarly, intervention studies using baked products prepared with buckwheat flour have demonstrated that consuming buckwheat daily for as little as 30 days reduces fasting blood glucose concentrations in individuals with diabetes [2,3]. The mechanisms underlying these beneficial effects on glycemia remain unknown.

We have previously documented glucose-lowering properties of a buckwheat concentrate (BWC) in both healthy rats and those with type 1 diabetes [4]. Specifically, intragastric administration of the BWC to healthy rats significantly reduced serum glucose concentrations during an intraperitoneal glucose tolerance test, while administration to rats with type 1 diabetes reduced fed state serum glucose concentrations by 14-16%. Initially, it was hypothesized that reduced glycemia was due to the insulin mimetic effects of d-chiro inositol, an inositol phosphoglycan naturally present in buckwheat with documented anti-hyperglycemic effects in animals [5,6]. However, follow-up studies revealed that treatment of H4IIE hepatoma cells with the BWC or isolated d-chiro inositol did not activate intermediates of the insulin signaling pathway [7] and thus the glucose-lowering effects of buckwheat occur independently from enhanced insulin signaling by d-chiro inositol. Interestingly, the BWC was shown to inhibit cellular glucose uptake in H4IIE cells, an unexpected finding given that reduction in blood glucose levels are typically associated with enhanced glucose uptake in peripheral tissues. However, inhibiting glucose uptake in enterocytes of the intestinal epithelium would reduce the amount of glucose absorbed into the bloodstream and

result in lower plasma glucose concentrations. To date, the inhibitory actions of buckwheat on cellular glucose uptake in intestinal cells have not been explored, the mechanism of inhibited glucose uptake remains to be elucidated, and the compound responsible for inhibition remains unknown. Thus, the objectives of the current study were to determine if a buckwheat extract (BWE) effectively inhibits glucose uptake, and also to determine if the inhibitory effects of buckwheat extend to Caco2 cells, a cell line commonly used to study intestinal absorption and transport [8]. The effect of buckwheat on pathways of glucose metabolism potentially inhibiting glucose uptake by an indirect mechanism was also explored.

## 5.3 Materials and Methods

*Preparation and fractionation of the buckwheat extract (BWE)*. The BWE was prepared from common buckwheat (*Fagopyrum esculentum*) variety Koto, which was provided by Minn-Dak Growers (Grand Forks, ND). The BWE and fractions of the BWE were provided by Jordan Betteridge.

*Cell culture.* Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548, passage 5-25) were cultured as previously described [7]. Briefly, cells were maintained in α-modified Minimum Essential Medium containing 10% fetal bovine serum, 2 mM glutamine, 25 µg/mL streptomycin, and 25 units/mL penicillin. Caco2 colorectal adenocarcinoma cells (American Type Culture Collection, HTB-37, passage 40-60) were maintained in α-modified Minimum Essential Media containing 20% fetal bovine serum, 2 mM glutamine, 25 µg/mL streptomycin, and 25 units/mL penicillin.

*$^{3}$H 2-deoxy-D-glucose (2-DOG) uptake.* $^{3}$H 2-DOG uptake assays were conducted as previously described [7]. Briefly, cells were cultured in 24-well tissue culture plates, grown to 80% confluence, and placed in serum-free media for 72 hours. On the day of an experiment, the media was aspirated from cells and replaced with pre-warmed

physiological salt solution (PSS; 145 mM NaCl, 5 mM KCl, 1 mM Hepes pH 7.4, 2.5 mM $Na_2HPO4$, 5 mM $MgCl_2$, 3 mM $CaCl_2$). Cells were treated with 0.25% v/v of the BWE or HPLC fractions of the BWE for 10 minutes (unless otherwise specified) at 37°C before addition of 5 mM cold 2-DOG plus 0.1 µCi/mL $^3$H 2-DOG (Perkin Elmer, Waltham, MA) for another 10 minutes. Uptake was terminated by removing media and rinsing 3 times with ice cold PSS. Cells were lysed in 0.1% SDS, with one aliquot reserved for liquid scintillation counting and one for determination of protein concentration using the BCA protein assay method.

*Caco2 transwell experiments.* $^3$H glucose transport experiments were carried out based on methods previously described [9]. Caco2 cells were seeded onto polyester Transwell® 0.4 µm pore size membrane supports (Corning, Tewksbury, MA) and allowed to differentiate for 21 days (see Appendix F for details of seeding and differentiation protocols). Integrity of the monolayer was assessed by measuring the electrical resistance of the epithelial cells in culture using a Millicell-ERS Voltohmmeter (Millipore, Billerica, MA). Only inserts with a resistance exceeding that of an unseeded membrane by 400 Ω were used. On the day of and experiment, the media was decanted from the Transwell inserts and cells were rinsed once with Hank's balance salt solution pH 7.4 (137 mM NaCl, 5.4 mM KCl, 0.25 mM $Na_2HPO4$, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$). Inserts were moved to new plates containing HBSS, and were then incubated with 0.25% or 0.5% v/v of the BWE in HBSS for 10 minutes before addition of 5 mM D-glucose plus 0.1 µCi/mL $^3$H D-glucose (Perkin Elmer, Waltham, MA). After 30 minutes, an aliquot of buffer was removed from the basolateral compartment and the radioactivity quantified by scintillation counting.

*Cyclic AMP (cAMP) measurements.* The concentration of cAMP in H4IIE and Caco2 cells treated with the BWE was measured using a cAMP EIA kit (Cayman Chemicals,

Ann Arbor, MI). Cells were lysed according the manufacturer's protocol, and lysates were diluted 1:4 for assays.

*Glucose production assays.* Glucose production in H4IIE cells was measured using a modification of a published protocol [10]. Briefly, H4IIE cells were seeded into the wells of a 96 well plate at a density of 100 000 cells per well. After incubation at 37°C for 3 hours, media was aspirated, the cells washed with sterile PBS and growth media replaced with glucose production media (glucose-free, phenol red-free DMEM plus 2 mM sodium pyruvate and 20 mM sodium lactate) before overnight incubation. The next day, glucose production media was refreshed and cells were treated for the specified times. Glucose production was assessed by measuring the glucose released into media using the Amplex Red Glucose Assay Kit (Invitrogen, Burlington, ON).

*Short hairpin RNA transductions.* H4IIE cells were transduced with SMARTvector 2.0 Lentivirus particles (Thermo Scientific, Ottawa, ON, Canada), packaged with a short hairpin RNA (shRNA) sequence targeting the MSK-1 gene (RPS6KA5) encoding the MSK-1 protein. Cells were infected according to the manufacturer's protocol in serum-free medium containing 3 µg/mL Polybrene, using a multiplicity of infection of 50 transducing units per cell. Cells transduced with a vector containing a non-targeting shRNA sequence served as transduction controls. Experiments were conducted 96 hours post-transduction, the time required for optimal knock-down of MSK-1 protein as determined during optimization experiments (data not shown).

*Western blotting.* Cells grown in 12-well culture dishes containing 2 mL serum-free medium were stimulated with the indicated compounds for 10 minutes, unless otherwise indicated. If used, inhibitors were added 10 minutes before the stimulating agents. After treatments were completed, cells were rinsed with PBS, and cellular protein extract preparation and Western blotting were carried out as previously described [11]. Primary antibodies for Western blotting (phospho-CREB ser$^{133}$, CREB, phospho-PKA, PKA,

phospho-PKA substrate (RRXS*/T*), MSK-1, phospho-MSK-1 ser$^{376}$, phospho-MSK-1 thr$^{581}$, PEPCK and G6Pase) were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA).

*Statistical analyses.* All analyses were performed using SAS statistical software (SAS v.9.1, SAS Institute Inc., Cary, NC). Statistical significance between treatment groups were determined using a mixed model analysis with a random intercept for the repetitions. Differences among groups were assessed using ANOVA with either estimate statements for individual comparisons of treatments versus the control or Duncan's multiple range test for post-hoc means testing. Differences were accepted as significant at $p<0.05$. Quantitative data are presented as mean ± SEM (n=3) unless otherwise indicated.

## 5.4 Results

*Inhibition of glucose uptake and phosphorylation of p42/44 ERK is unique to common buckwheat.* We have previously reported two major bioactivities present in a concentrate prepared from common buckwheat of the Koto variety in H4IIE hepatoma cells: inhibition of 2-DOG uptake and phosphorylation of p42/44 ERK [7]. From this previous study, we were unable to determine the identity of the compound(s) responsible for these activities. To determine if these bioactivities would be present in an extract, Koto buckwheat was subjected to a solvent extraction procedure. Concurrently, the other major buckwheat species, Fagopyrum *tartaricum* (tartary buckwheat), was also subjected to this extraction to determine if the previously-documented bioactivities were also present in other buckwheat species. BWE, along with the original BWC, was used to treat H4IIE cells for Western blot analyses and $^{3}$H 2-DOG uptake assays. Treating H4IIE cells with 0.25% and 0.5% v/v of the Koto extract stimulated phosphorylation of p42/44 ERK and inhibited $^{3}$H 2-DOG uptake, similar to that originally observed upon

treatment with 0.25% v/v of the BWC (Figure 5.1A and 5.1B). However, treatment of H4IIE cells with either 0.25% or 0.5% v/v of the tartary buckwheat extract failed to stimulate phosphorylation of p42/44 ERK or inhibit $^{3}$H 2-DOG uptake (Figure 5.1A and 5.1B).

*Inhibition of 2-DOG uptake in H4IIE cells treated with buckwheat is reversible and likely competitive.* To test if inhibition of glucose uptake upon treatment with the BWE is reversible, $^{3}$H 2-DOG uptake experiments were conducted with the addition of a 10 minute wash between BWE treatment and incubation with 2-DOG. Inclusion of a set of cells treated with the BWE for 2 minutes prior to addition of 2-DOG demonstrated that this shorter incubation with the BWE was sufficient to inhibit 2-DOG uptake (Figure 5.1C). In cells treated with BWE for both 2 minutes and 10 minutes, the wash step resulted in levels of 2-DOG uptake that were not different than those observed in cells not exposed to the BWE (Figure 5.1C).

Determining the exact nature of inhibited 2-DOG uptake (i.e. competitive versus non-competitive) is difficult because the identity and therefore concentration of the inhibitory bioactive currently remains unknown. However, 2-DOG uptake experiments with increasing concentrations of 2-DOG were conducted to provide a clue as to the nature of inhibition. Significant inhibition after incubation with the BWE was observed when cells were treated with $^{3}$H 2-DOG alone (the equivalent of 5 fM 2-DOG) and with 0.5 mM 2-DOG, but not with higher concentrations of 2-DOG such as 5 mM and 25 mM (Figure 5.1D), suggesting competitive inhibition. However, follow-up kinetic studies are required to definitely determine the type of inhibition induced by the BWE.

*Buckwheat reduces overall glucose uptake and transepithelial glucose transport in Caco2 cells.* To determine if the BWE inhibits 2-DOG uptake in other cells, Caco2 cells were pre-treated with the BWE for 10 minutes before treatment with 5 mM 2-DOG. Similar to what has been observed in H4IIE cells, 2-DOG uptake was 45% lower in

Caco2 cells treated with the BWE compared to those treated with 2-DOG alone (Figure 5.2A). Post-prandial increases in blood glucose concentrations reflect the amount of glucose transported across the intestinal epithelium into the bloodstream. To relate inhibition of glucose uptake by the BWE in Caco2 cells to reduced transepithelial glucose transport, we conducted glucose transport assays in differentiated Caco2 monolayers cultured on Transwell® permeable supports. In monolayers pre-treated with the BWE, glucose transport was reduced significantly to 65% of that observed in untreated control cells; 0.5% v/v of BWE significantly reduced glucose transport to 49% of that observed in untreated control cells (Figure 5.2B).

*Glucose uptake inhibitory activity of the BWE is separable by HPLC fractionation*. HPLC fractionation of the BWE was performed and fractions were used to treat H4IIE cells for glucose uptake assays. HPLC fraction 6 was the only fraction that significantly inhibited 2-DOG uptake to a similar degree as the BWE (Figure 5.2C).

*Buckwheat stimulates glucose production and upregulates protein levels of gluconeogenic enzymes*. GLUT2 is the predominant glucose transporter expressed in primary hepatocytes, but is also present in H4IIE cells [12]. Due to the low affinity, high-capacity kinetics of GLUT2 and its constitutive expression in the plasma membrane, the direction of glucose movement through GLUT2 is determined by relative intracellular versus extracellular glucose concentrations, with glucose moving from higher to lower concentrations. Therefore, the inhibitory actions of buckwheat on glucose uptake may be indirect and involve stimulation of glucose production and increases in intracellular glucose concentrations, consequently blocking cellular glucose entry. To test for such an effect, the amount of glucose released into culture medium in BWE-treated H4IIE cells cultured in glucose-free medium was measured. Treating cells with insulin ranging from 1 to 250 nM for 10 minutes resulted in an almost complete suppression of glucose production (Figure 5.3A). Conversely, cells treated with the BWE for 10 minutes

produced a significant 5-fold increase in glucose levels compared to untreated cells, at all doses tested. Time course treatment with the BWE showed that after the initial elevation in glucose production observed after 10 minutes of treatment, glucose production started to decline (Figure 5.3B). Interestingly, a second elevation in glucose production was observed after 4 hours of treatment (Figure 5.3B).

Immediate increases in glucose production by hepatocytes involves enhanced activity of glycogen phosphorylase for glycogen breakdown, whereas long-term control of glucose production requires increased levels of glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), two rate-limiting enzymes of gluconeogenesis. To test the potential for buckwheat to modulate long-term glucose production, H4IIE cells were treated with the BWE for up to 24 hours, and protein levels of G6Pase and PEPCK in cells were measured using Western blotting (Figure 5.4A). Protein levels of G6Pase were significantly higher 8 and 16 hours after treatment and returned to baseline levels 24 hours after treatment. On the other hand, protein levels of PEPCK steadily increased over 8 and 16 hours, finally reaching maximal levels 24 hours after treatment. Furthermore, levels of both G6Pase and PEPCK were elevated to a similar extent as when cells were treated with the adenylate cyclase activator forskolin (Figure 5.4B and 5.4C).

*BWE treatment stimulates phosphorylation of cAMP response element-binding protein (CREB) and other protein kinase A (PKA) substrates independent of increases in cAMP and PKA phosphorylation.* CREB is one of several transcription factors influencing transcription of PEPCK and G6Pase [13]. Consistent with results demonstrating higher levels of both G6Pase and PEPCK, CREB phosphorylation was stimulated in H4IIE cells treated with the BWE (Figure 5.5A). Treatment of cells with HPLC fractions of the BWE revealed that CREB phosphorylating activity did not separate to a single fraction;

however, phosphorylation was lowest in fraction 6, the fraction responsible for inhibiting 2-DOG uptake (Figure 5.5A).

The best known pathway of CREB phosphorylation involves activation of G protein-coupled receptors, leading to increased intracellular cAMP and consequent activation of PKA [13]. However, phosphorylation of PKA was not observed in H4IIE cells treated with the BWE for up to one hour (Figure 5.5B), suggesting that PKA may not be the kinase responsible for phosphorylation of CREB after treatment with the BWE. Consistent with these results, cAMP concentrations were not affected by the BWE in both H4IIE and Caco2 cells, although treatment with the adenylate cyclase activator forskolin resulted in significantly higher cAMP concentrations in both cell lines (Figure 5.5C).

Phosphorylation of CREB in the absence of PKA phosphorylation prompted us to probe for other PKA substrates that might be phosphorylated in response to the BWE. Western blotting using an antibody directed against peptides containing the PKA phosphorylation motif detected 3 immunoreactive bands of approximately 33, 85 and 185 kDa that were significantly higher in intensity after treatment with the BWE (Figure 5.5D). Interestingly, higher intensity of the 33 and 85 kDa bands were observed after 30 minutes of treatment, whereas 1 hour of treatment was required to achieve significantly higher intensity of the 185 kDa band.

*H89 prevents phosphorylation of CREB induced by the BWE*. To determine if elevated PKA activity accounted for higher CREB phosphorylation in response to BWE treatment, cells were pre-treated with KT5720 and H89, two chemical inhibitors of PKA, before treatment with the BWE. Pre-treatment of cells with KT5720 did not diminish phosphorylation of CREB in response to the BWE (Figure 5.6A); however, CREB phosphorylation was markedly reduced in cells pre-treated with H89 (Figure 5.6C). H89 inhibits several other kinases in addition to PKA, including p90 ribosomal S6 kinase

(p90RSK) and mitogen and stress activated protein kinase (MSK-1), both of which have been implicated in ser$^{133}$ phosphorylation of CREB [13-15]. Pre-treatment of cells with SL0101, an inhibitor of p90RSK activity, did not prevent phosphorylation of CREB in response to the BWE (Figure 5.6B), suggesting MSK-1 as the CREB kinase responsible activated by the BWE. To confirm this, H4IIE cells were infected with lentivirus containing an shRNA construct directed against the MSK-1 gene. These cells containing the MSK-1 knockdown were treated with BWE and Western blotting was performed to assess CREB phosphorylation in cells lacking the MSK-1 protein. As opposed to effects observed with H89, silencing of MSK-1 did not prevent the stimulation of CREB phosphorylation upon buckwheat treatment (Figure 5.5D).

Although MSK-1 silencing did not prevent CREB phosphorylation by the BWE, treatment of H4IIE cells with the BWE resulted in significantly higher levels of phosphorylated MSK-1 on thr$^{581}$, but not ser$^{389}$ (Figure 5.7A), a response similar to that observed when cells were treated with the PKC activator ingenol [16]. This phosphorylation of MSK-1 was dependent on the activity of both p42/44 ERK and protein kinase C, as evidenced by a reduction in phosphorylated MSK-1 thr$^{581}$ when cells were treated with PD98059 and Go6796 (Figure 5.7B). However, inhibition of MSK-1 activity was not linked to the inhibitory effects of the BWE on glucose uptake, as treating H4IIE cells with H89 either before or after the BWE did not prevent inhibition of 2-DOG uptake (Figure 5.7C).

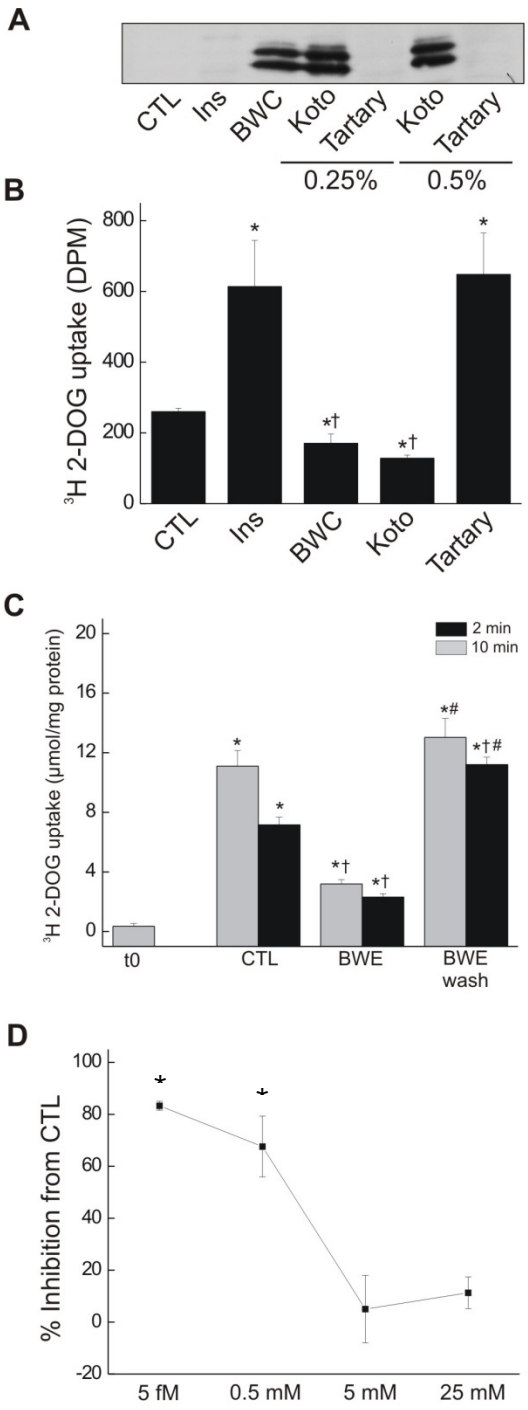


**Figure 5.1 Inhibition of 2-deoxy-D-glucose (2-DOG) uptake by common buckwheat is reversible and likely competitive.** (A) H4IIE cells were treated with 250 nM insulin (Ins), 0.25% v/v buckwheat concentrate (BWC), 0.25% or 0.5% Koto buckwheat extract (Koto) or 0.25% or 0.5% tartarty buckwheat extract (tartary) for 10 minutes, with untreated cells serving as the control (CTL). Phosphorylation of p42/44 ERK was assessed using Western blotting. (B) H4IIE cells were treated with 250 nM Ins, 0.25% v/v BWC, 0.25% v/v Koto buckwheat extract or 0.25% v/v tartary buckwheat extract for 10 minutes before addition of 0.1 µCi/mL $^3$H 2-DOG. * denotes significantly different from CTL ($p<0.05$); † denotes significantly different from Ins ($p<0.05$). (C) H4IIE cells were treated with 0.25% v/v buckwheat extract (BWE) for 2 minutes (black bars) or 10 minutes (gray bars) before addition of 0.5 mM 2-DOG/0.1 µCi/mL $^3$H 2-DOG for 10 minutes. One set of cells was washed for 10 minutes after incubation with BWE before addition of 2-DOG (BWE wash). * denotes significantly different from the start of the 10 minute incubation with 2-DOG (t0; $p<0.05$); † denotes significantly different from untreated control (CTL) cells ($p<0.05$); # denotes significantly different from BWE ($p<0.05$). (D) H4IIE cells were treated with 0.25% v/v BWE before addition of the indicated concentrations of 2-DOG. * denotes significantly different from cells not treated

with BWE ($p<0.05$). For panels B, C and D, data are presented as mean ± SEM (n=3).

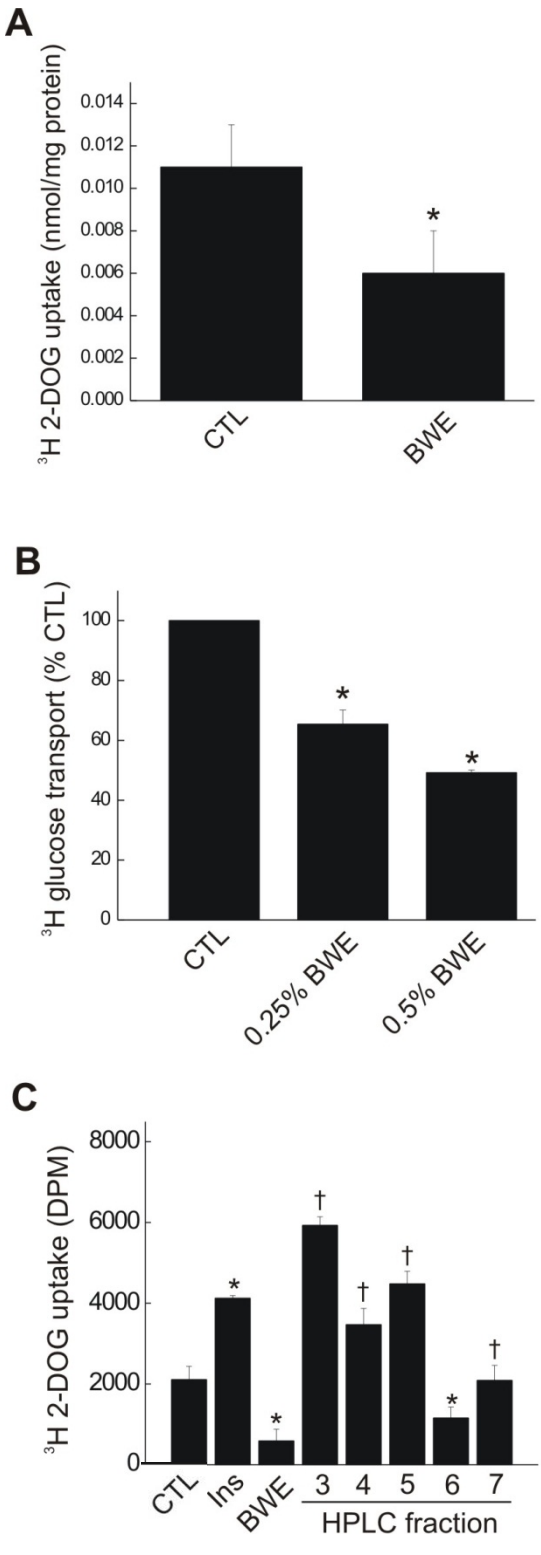


**Figure 5.2 Buckwheat inhibits 2-deoxy-D-glucose (2-DOG) uptake and glucose transport in Caco2 cells.** (A) Caco2 cells were treated with 0.25% v/v buckwheat extract (BWE) for 10 minutes prior to addition of 5 mM 2-DOG/0.1 µCi/mL $^3$H 2-DOG for 10 minutes. * denotes significantly different from untreated control (CTL) cells ($p<0.05$). (B) Monolayers of Caco2 cells were grown on Transwell® permeable supports, and 0.25% or 0.5% v/v BWE was added to the apical side 10 minutes before exposure to 5 mM D-glucose/0.1 µCi $^3$H D-glucose for 30 minutes. * denotes significantly different from untreated monolayers (CTL; $p<0.05$). (C) H4IIE cells were treated with 250 nM insulin (Ins) or 0.25% v/v of the BWE or HPLC fractions of the BWE for 10 minutes prior to the addition of 0.1 µCi/mL $^3$H 2-DOG for 10 minutes. * denotes significantly different from CTL ($p<0.05$); † denotes significantly different from BWE ($p<0.05$). For all panels, data are presented as mean ± SEM (n=3).

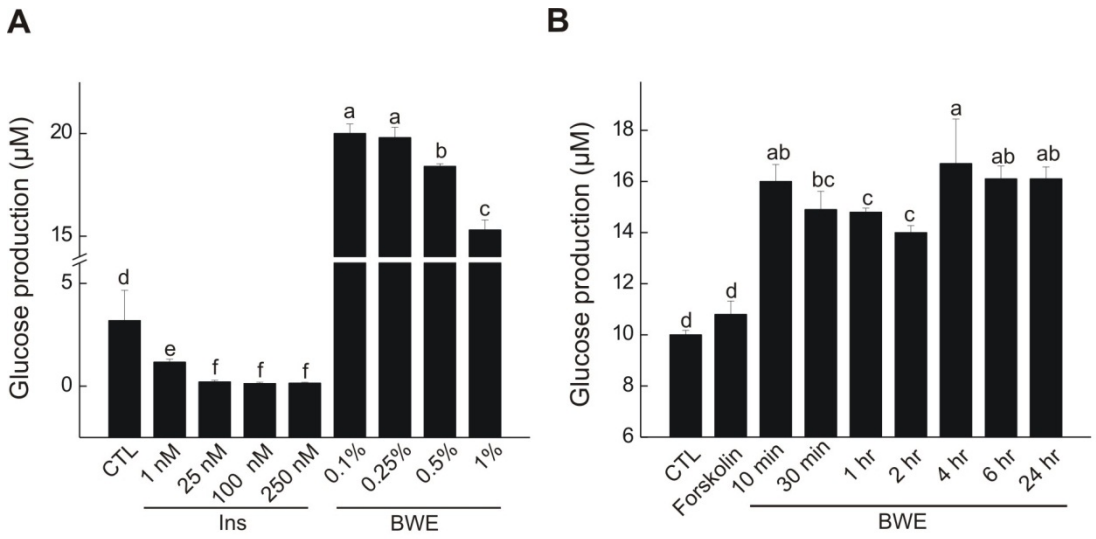


**Figure 5.3 Buckwheat stimulates glucose production in H4IIE cells.** H4IIE cells were treated with the indicated concentrations of insulin (Ins) or buckwheat extract (BWE) for 10 minutes (A) or 0.25% v/v BWE for the indicated times (B). Untreated cells served as a control (CTL). Means with different letters are significantly different ($p<0.05$). Data are presented as mean ± SEM (n=6).

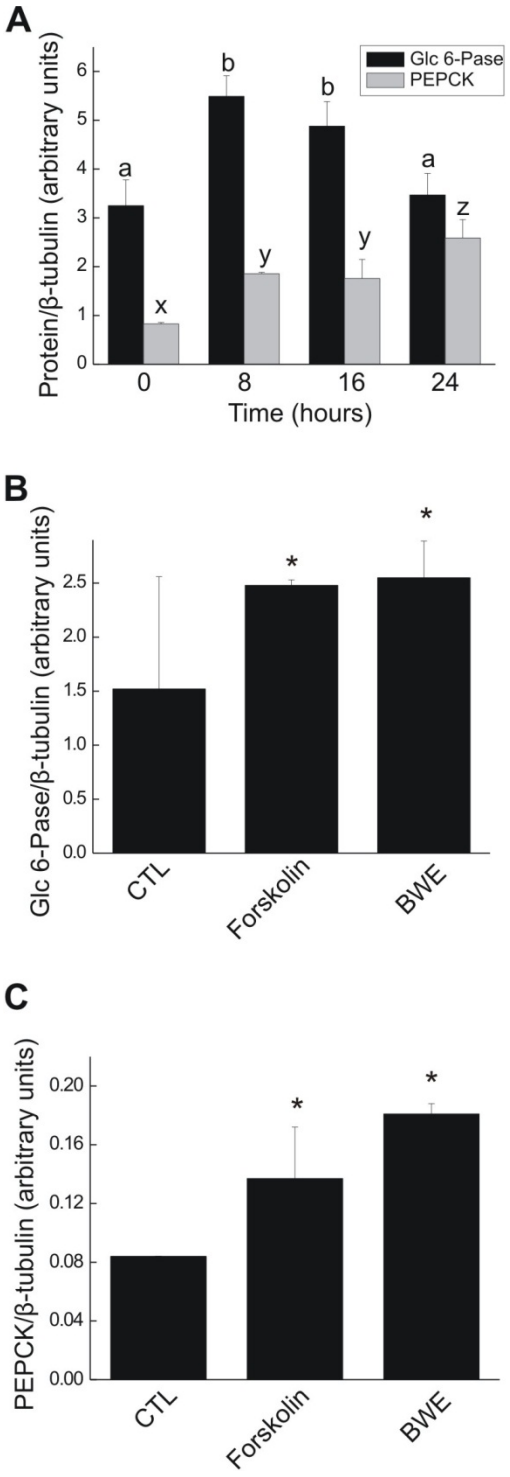


**Figure 5.4 Buckwheat enhances protein levels of gluconeogenic enzymes.** (A) H4IIE cells were treated with the buckwheat extract (BWE) for the indicated times and lysates used for Western blotting with antibodies directed against G6-Pase (black bars) or PEPCK (grey bars). Means with different letters are significantly different ($p<0.05$); a and b are used for comparisons among G6-Pase groups; x, y and z are used for comparisons among PEPCK groups. (B) and (C) H4IIE cells were treated with $10^{-5}$ M forskolin or 0.25% v/v of the BWE for 16 hours and lysates used for Western blotting with antibodies directed against G6-Pase (B) or PEPCK (C). * denotes significantly different from untreated control (CTL) cells ($p<0.05$). For all panels, data are presented as means ± SEM (n=3 per group).

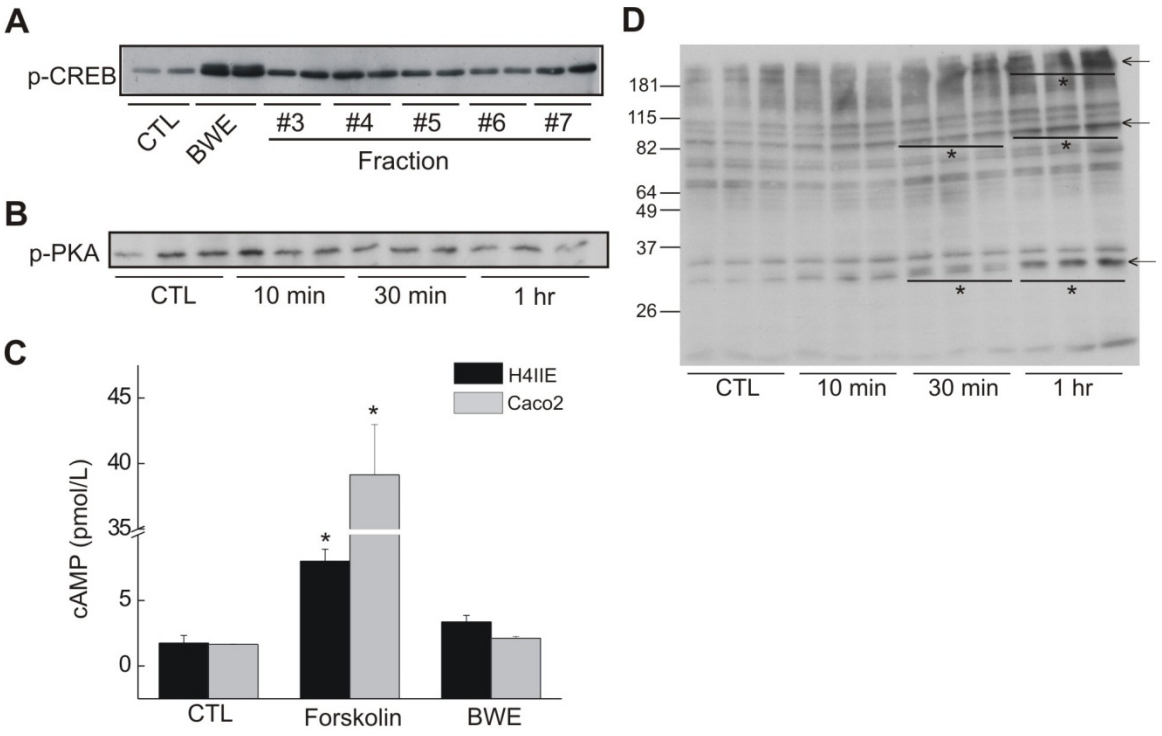


**Figure 5.5 Buckwheat phosphorylates PKA substrates and CREB, but does not increase cAMP concentrations or increase phosphorylation of PKA.** (A) H4IIE cells were treated with 0.25% buckwheat extract (BWE) or HPLC fractions of the BWE for 10 minutes and lysates used for Western blotting with antibodies directed against phospho-CREB ser$^{133}$. (B) H4IIE cells were treated with 0.25% v/v BWE for 10 minutes, 30 minutes or 1 hour, and cell lysates used for Western blotting with antibodies directed against phosphorylated PKA. (C) H4IIE and Caco2 cells were treated with 0.25% v/v BWE or $10^{-5}$ M forskolin for 10 minutes, and cell lysates used for measurements of cAMP concentrations using a commercial EIA kit. * denotes significantly different from untreated control (CTL) cells ($p<0.05$). Data are presented as mean ± SEM (n=3). (D) H4IIE cells were treated with 0.25% v/v BWE for 10 minutes, 30 minutes or 1 hour, and cell lysates subjected to Western blotting with antibodies directed against phosphorylated PKA substrates. * denotes significantly different from untreated control (CTL) cells ($p<0.05$).

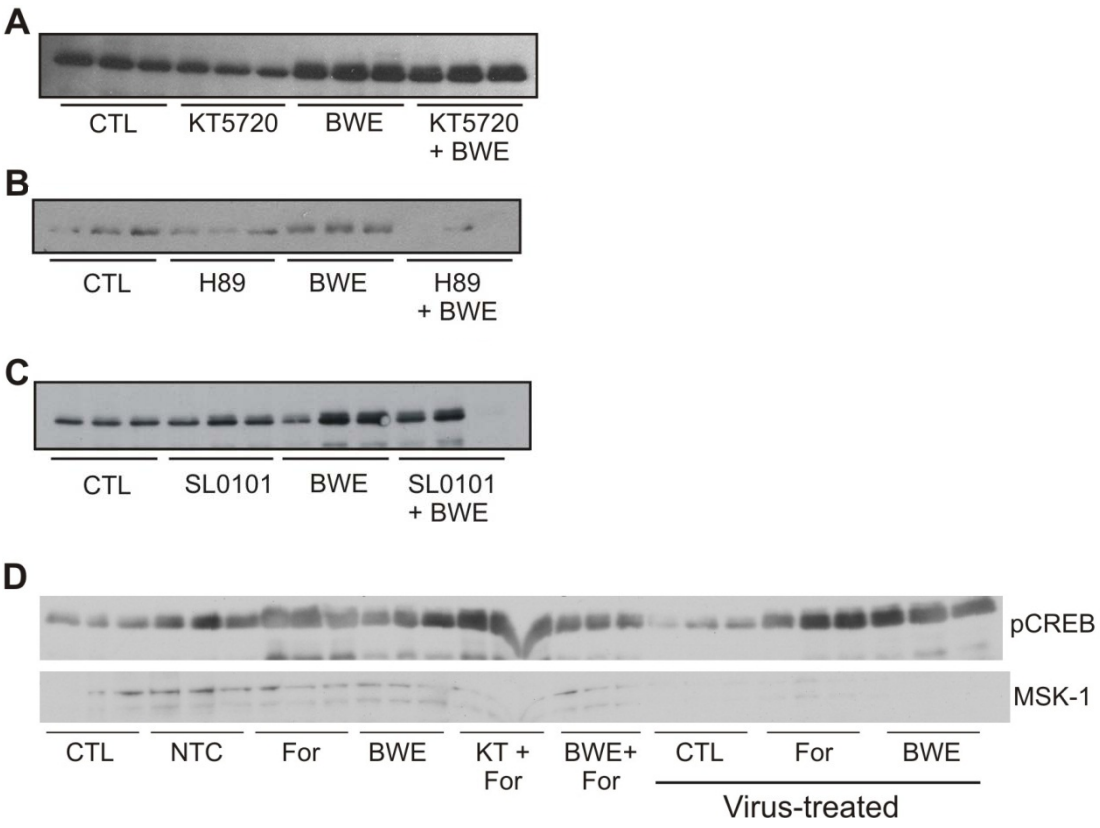


**Figure 5.6 Phosphorylation of CREB in response to buckwheat is sensitive to the chemical inhibitor H89.** (A, B and C) H4IIE cells were treated with $10^{-5}$ M KT5720 (inhibits PKA), $10^{-5}$ M H89 (inhibits MSK-1) or $10^{-5}$ M SL0101 (inhibits p90 RSK) for 10 minutes prior to addition of the buckwheat extract (BWE) for an additional 10 minutes. (D) H4IIE cells were treated with $10^{-5}$ M KT5720 (KT) or 0.25% v/v BWE for 10 minutes before or after addition of $10^{-5}$ M forskolin (For) or BWE 0.25% BWE for 10 minutes. H4IIE cells infected with an MSK-1 shRNA-containing lentivirus (virus-treated) were also treated with forskolin and the BWE for 10 minutes. Untreated cells (CTL) and cells treated with a non-targeting shRNA lentivirus (NTC) served as controls. Phosphorylation of CREB $ser^{133}$ was assessed using Western blotting. Representative blots (n=3) are shown.

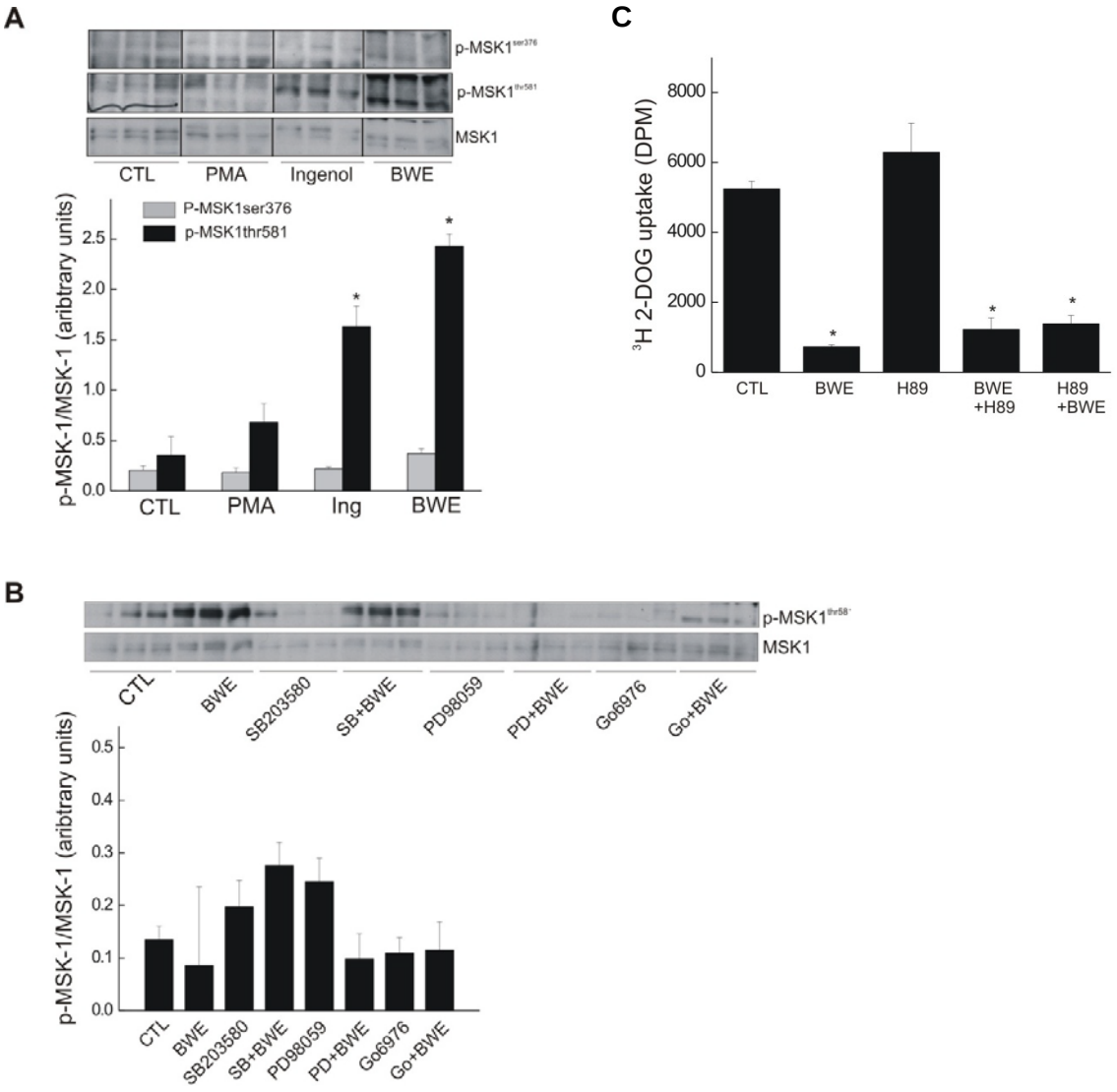


**Figure 5.7 Phosphorylation of MSK-thr$^{581}$ by buckwheat is dependent on p42/44 ERK and PKC.** (A) H4IIE cells were treated with $10^{-9}$ M phorbol-12-myristate-13-acetate (PMA), $10^{-6}$ M ingenol or 0.25% v/v buckwheat extract (BWE) for 10 minutes, and lysates used for Western blotting with antibodies directed against phosphorylated MSK-1 on ser$^{376}$ (grey bars) or thr$^{581}$ (black bars). (B) H4IIE cells were treated with $10^{-5}$ M SB203580, (inhibits p38 MAPK), $10^{-6}$ M PD98059 (inhibits p42/44 ERK signaling) or $10^{-5}$ M Go6976 (inhibits PKC) for 10 minutes prior to addition of the BWE for an additional 10 minutes. Phosphorylation of MSK1 thr$^{581}$ was assessed using Western blotting. Representative blots (n=3) are shown. (C) H4IIE cells were treated with $10^{-5}$ M H89 or 0.25% v/v BWE for 10 minutes, or the BWE for 10 minutes followed by H89 for 10 minutes (BWE+H89), or H89 for 10 minutes followed by the BWE for 10 minutes (H89+BWE), after which 0.1 µCi /mL $^{3}$H 2-DOG was added for 10 minutes. * denotes significantly different from untreated control cells (CTL). For all panels, * denotes significantly different from untreated control cells (CTL). Data are presented as mean ± SEM (n=3).

## 5.5 Discussion

We have previously demonstrated that a concentrate produced from common buckwheat inhibits glucose uptake in H4IIE cells [7]. In the current study, we show that an extract produced from common buckwheat also possesses this glucose uptake inhibitory activity, which is unique to common buckwheat. The BWE also inhibited glucose uptake in Caco2 cells and reduced transepithelial glucose transport in Caco2 monolayers. H4IIE cells express only the GLUT1 and GLUT2 facilitative glucose transporter isoforms (Chapter 6); therefore reduced glucose uptake by buckwheat in this cell line indicates inhibition of facilitative glucose transport mechanisms. Buckwheat is a source of numerous flavonoids, including rutin, orientin, vitexin, quercetin, isovitexin, quercetrin and isorientin [17], several of which have been shown to inhibit facilitative glucose uptake through direct, non-competitive inhibition of GLUT2 [9]. Other non-glycosylated polyphenols have been shown to inhibit glucose uptake under both sodium-free and sodium-dependent conditions [18], suggesting these compounds can inhibit active glucose transport in addition to facilitative glucose transport. From our previous studies, we have determined that neither rutin nor its aglycone form quercetin is the compound in buckwheat responsible for inhibitory actions on glucose uptake [7], and follow-up studies have also shown that other flavonoids such as myricetin, keamferol, as well as isoflavones such as diadzein and genistein and the anthocyanidin delphinidin do not inhibit glucose uptake under the conditions used in our experiments (data not shown). Therefore, the identity of the compound responsible for inhibition of glucose uptake remains unknown.

HPLC fractionation of the BWE showed that fraction 6 was the only fraction which significantly inhibited glucose uptake to a similar degree as that of the entire BWE. These data suggest that this approach can be used in future studies to identify the compound(s) in buckwheat capable of inhibiting facilitative glucose uptake. Glucose-

lowering effects of an extract prepared from common buckwheat have been reported by other groups [19], although the proposed mechanism in these studies involved inhibition of intestinal sucrase and reduced carbohydrate digestion. Therefore, common buckwheat may contain multiple compounds working through distinct mechanisms involving altered intestinal carbohydrate digestion and absorption to reduce blood glucose concentrations [19]. Tartary buckwheat, the other major buckwheat species has also shown glucose-lowering properties in *vivo* [20]; however, the current study has shown that tartary buckwheat does not inhibit cellular glucose uptake. Species-specific differences in production of phytochemicals likely accounts for this difference.

Several attempts to determine the signaling pathways involved in the inhibition of glucose uptake by buckwheat with the use of chemical inhibitors have ruled out involvement of p42/44 ERK, p38 MAPK, AMP-activated protein kinase, PI3Kγ, PKA, mTOR and PKC signaling [1, data not shown]. An alternative mechanism to explain inhibited glucose uptake may involve indirect inhibition of glucose movement through GLUT2, the predominant glucose transporter expressed in H4IIE cells [12]. Due to low affinity, high capacity kinetics of GLUT2, the direction of glucose flux depends on intracellular versus extracellular glucose concentrations, with the direction of movement dictated by the concentration gradient [21]. In the current study, treatment with the BWE did enhance glucose production, which may have blocked glucose entry as glucose produced by the cell was exiting the cell. However, H4IIE cells also express GLUT1 (Chapter 6) which has high affinity, low capacity kinetic characteristics and would likely have been able to transport glucose into the cell even if GLUT2 was prevented from doing so.

Short-term increases in glucose production result from glycogen breakdown through increased activity of glycogen phosphorylase. Although we did not measure the effects of buckwheat on glycogen phosphorylase activity, we observed up-regulation of

G6Pase and PEPCK levels, suggesting that buckwheat affects longer-term glucose production by stimulating gluconeogenesis. One of the major regulators of gluconeogenesis is CREB which, upon phosphorylation, promotes transcription of G6Pase and PEPCK [22]. Activation of G-protein coupled receptors, increases in cAMP and consequent activation of PKA is the most-well characterized pathway of CREB activation [22,23]. However, CREB phosphorylation after buckwheat treatment in the current study was not accompanied by increased cAMP or phosphorylation of PKA, suggesting activation of another CREB kinase. Contrary to results using various inhibitors of several CREB kinases, MSK-1 gene silencing experiments did not support MSK-1 as the CREB kinase stimulated by buckwheat, despite increased phosphorylation of MSK-1 in cells treated with buckwheat. Phosphorylation of MSK-1 by buckwheat was dependent on both PKC- and p42/44 ERK, but the functional significance of this activation remains unknown. Future studies on the mitogenic effects of buckwheat are needed to determine the functional significance of activation of this pathway. However, based on results from the current study, activation of MSK-1 is not linked to inhibitory activity of buckwheat on cellular glucose uptake.

One of the major limitations of the current study is that the identities of the compounds responsible for inhibited glucose uptake remain unknown. Without knowledge of the identities and therefore concentrations of these compounds, we are unable to conduct mechanistic kinetic studies to definitely state the nature of inhibition. We demonstrated that buckwheat inhibits both facilitative and active glucose transport but are still unaware of the mechanism involved, although it likely does not involve altered signaling pathways regulating glucose uptake. Studies using individual glucose transporters expressed in *Xenopus laevis* oocytes would determine if direct inhibition of glucose transporters in involved, as has been observed with various flavonoids. Additionally, we cannot conclude that inhibition of facilitative and active glucose transport

*in vitro* would translate to inhibition *in vivo*; future experiments using everted intestinal sleeves or brush border vesicle preparations would be required to determine if the inhibitory effects we observed are also present in intestinal systems *in vivo*.

In conclusion, common buckwheat contains several compounds with the ability to inhibit facilitative glucose transport via a mechanism independent of several signaling pathways regulating glucose metabolism. Buckwheat influences other aspects of glucose metabolism, including glucose production and levels of gluconeogenic enzymes. Identifying the compounds responsible for inhibited glucose uptake could facilitate the development of food products enriched in these compounds, potentially increasing the availability of dietary agents for effective glycemic management.

## 5.6 Literature Cited

[1] Zhang, H.W., Zhang, Y.H., Lu, M.J., Tong, W.J., and Cao, G.W. (2007). Comparison of hypertension, dyslipidaemia and hyperglycaemia between buckwheat seed-consuming and non-consuming Mongolian-Chinese populations in Inner Mongolia, China. *Clin. Exp. Pharmacol. Physiol*. 34, 838-44.

[2] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia and hyperlipidemia. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, pp 458-64.

[3] Wang, J., Liu, Z., Fu, X., and Run, M. (1992). A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, 465-7.

[4] Kawa, J.M., Taylor, C.G., and Przybylski, R. (2003). Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. *J. Agric. Food Chem.* 51, 7287-91.

[5] Fonteles, M.C., Almeida, M.Q., and Larner, J. (2000). Antihyperglycemic effects of 3-O-methyl-D-chiro-inositol and D-chiro-inositol associated with manganese in streptozotocin diabetic rats. *Horm. Metab. Res.* 32, 129-32.

[6] Ortmeyer, H.K., Bodkin, N.L., Lilley, K., Larner, J., and Hansen, B.C. (1993). Chiroinositol deficiency and insulin resistance. I. Urinary excretion rate of chiroinositol is

directly associated with insulin resistance in spontaneously diabetic rhesus monkeys. *Endocrinology* 132, 640-5.

[7] Curran, J.M., Stringer, D.M., Wright, B., Taylor, C.G., Przybylski, R., and Zahradka, P. (2010). Biological response of hepatomas to an extract of Fagopyrum esculentum M. (buckwheat) is not mediated by inositols or rutin. *J. Agric. Food Chem.* 58, 3197-204.

[8] Stenberg, P., Norinder, U., Luthman, K., and Artursson, P. (2001). Experimental and computational screening models for the prediction of intestinal drug absorption. *J. Med. Chem.* 44, 1927-37.

[9] Kwon, O., Eck, P., Chen, S., Corpe, C.P., Lee, J.H., Kruhlak, M., and Levine, M. (2007). Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J.* 21, 366-77.

[10] de Raemy-Schenk, A.M., Trouble, S., Gaillard, P., Page, P., Gotteland, J.P., Scheer, A., Lang, P., and Yeow, K. (2006). A cellular assay for measuring the modulation of glucose production in H4IIE cells. *Assay Drug Dev. Technol.* 4, 525-33.

[11] Yau, L., Lukes, H., McDiarmid, H., Werner, J., and Zahradka, P. (1999). Insulin-like growth factor-I (IGF-I)-dependent activation of pp42/44 mitogen-activated protein kinase occurs independently of IGF-I receptor kinase activation and IRS-1 tyrosine phosphorylation. *Eur. J. Biochem.* 266, 1147-57.

[12] Mardones, L., Ormazabal, V., Romo, X., Jana, C., Binder, P., Pena, E., Vergara, M., and Zuniga, F.A. (2011). The glucose transporter-2 (GLUT2) is a low affinity dehydroascorbic acid transporter. *Biochem. Biophys. Res. Commun.* 410, 7-12.

[13] Altarejos, J.Y. and Montminy, M. (2011). CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. *Nat. Rev. Mol. Cell Biol.* 12, 141-51.

[14] Vermeulen, L., Vanden Berghe, W., Beck, I.M., De Bosscher, K., and Haegeman, G. (2009). The versatile role of MSKs in transcriptional regulation. *Trends Biochem. Sci.* 34, 311-8.

[15] Frodin, M. and Gammeltoft, S. (1999). Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol. Cell. Endocrinol.* 151, 65-77.

[16] Kedei, N., Lundberg, D.J., Toth, A., Welburn, P., Garfield, S.H., and Blumberg, P.M. (2004). Characterization of the interaction of ingenol 3-angelate with protein kinase C. *Cancer Res.* 64, 3243-55.

[17] Kreft, S., Knapp, M., and Kreft, I. (1999). Extraction of rutin from buckwheat (Fagopyrum esculentumMoench) seeds and determination by capillary electrophoresis. *J. Agric. Food Chem.* 47, 4649-52.

[18] Johnston, K., Sharp, P., Clifford, M., and Morgan, L. (2005). Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett.* 579, 1653-7.

[19] Hosaka, T., Nii, Y., Tomotake, H., Ito, T., Tamanaha, A., Yamasaka, Y., Sasaga, S., Edazawa, K., Tsutsumi, R., Shuto, E., Okahisa, N., Iwata, S., and Sakai, T. (2011). Extracts of common buckwheat bran prevent sucrose digestion. *J. Nutr. Sci. Vitaminol. (Tokyo)* 57, 441-5.

[20] Yao, Y., Shan, F., Bian, J., Chen, F., Wang, M., and Ren, G. (2008). D-chiro-inositol-enriched tartary buckwheat bran extract lowers the blood glucose level in KK-Ay mice. *J. Agric. Food Chem.* 56, 10027-31.

[21] Gould, G.W., Thomas, H.M., Jess, T.J., and Bell, G.I. (1991). Expression of human glucose transporters in Xenopus oocytes: kinetic characterization and substrate specificities of the erythrocyte, liver, and brain isoforms. *Biochemistry* 30, 5139-45.

[22] Wynshaw-Boris, A., Short, J.M., Loose, D.S., and Hanson, R.W. (1986). Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. I. Multiple hormone regulatory elements and the effects of enhancers. *J. Biol. Chem.* 261, 9714-20.

[23] Herzig, S., Long, F., Jhala, U.S., Hedrick, S., Quinn, R., Bauer, A., Rudolph, D., Schutz, G., Yoon, C., Puigserver, P., Spiegelman, B., and Montminy, M. (2001). CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 413, 179-83.

# Chapter 6: Expression of GLUT3 in Clone 9 liver cells and its translocational control by insulin

Danielle M. Stringer[1,3] , Carla G. Taylor[1,2,3] & Peter Zahradka[1,2,3]

Departments of [1]Human Nutritional Sciences and [2]Physiology University of Manitoba, Winnipeg, Manitoba, Canada

[3]Canadian Centre for Agri-food Research in Health and Medicine, St. Boniface Research Centre, Winnipeg, Manitoba, Canada

## 6.1 Abstract

Clone 9 cells have been reported to express only the GLUT1 facilitative glucose transporter; however, previous studies have not examined Clone 9 cells for GLUT3 expression. The current study sought to determine the expression profile of glucose transporters in Clone 9 cells. GLUT3 was detected at both the gene and protein level. Glucose transport in Clone 9 cells was insulin-sensitive in a concentration-dependent manner, concomitant with the presence of GLUT3 in the plasma membrane after insulin treatment. These results contrast previous reports that Clone 9 cells exclusively express GLUT1 and suggest GLUT3 is an insulin-sensitive glucose transporter.

## 6.2 Introduction

Glucose is a vital cellular fuel source that requires assistance from integral membrane transporter proteins to cross the plasma membrane and enter the cell. Mammalian glucose transporters belong to one of two classes originating from separate gene families: sodium-dependent active glucose transporters (SGLTs) and facilitative glucose transporters, also known as GLUT transporters [1]. GLUT isoforms share significant structural similarity but differ in kinetic properties and tissue distribution, and their cell-specific expression is a product of both the metabolic function and glucose requirements of the particular cell type and the unique kinetics of the individual transporters [2].

The Clone 9 liver cell line is an untransformed, epithelial-like cell line first isolated from the livers of healthy 6 to 8 week old male Sprague-Dawley rats [3]. Clone 9 cells have been used extensively to study regulation of the GLUT1 facilitative glucose transporter isoform, as these cells are cited in numerous publications as expressing exclusively GLUT1 under both basal and stimulated conditions [4,5]. However, the claim of exclusive GLUT1 expression in Clone 9 cells is based on initial Northern blotting experiments using cDNA probes for what were known at that time as the erythrocyte/rat brain/HepG2 cell transporter (later termed GLUT1), the rat muscle/fat cell transporter (later termed GLUT4) and the rat liver transporter (later termed GLUT2). It was determined from these studies that Clone 9 cells express GLUT1 but not GLUT2 or GLUT4 [5-7]. Over time, this initial observation has been interpreted to mean that GLUT1 is the only glucose transporter expressed in these cells [4,5,8]. However, Clone 9 cells have not been assessed for expression of GLUT3, the facilitative glucose transporter isoform expressed predominantly in neurons, and to a lesser extent, in carcinoma cell lines in kidney, colon and placenta [9].

In the current study, several commonly used cell lines, including Clone 9 cells, were screened for the presence of both facilitative glucose transporter isoforms (GLUT1, GLUT2, GLUT3 and GLUT4) and active glucose transporter isoforms (SGLT-1 and SGLT-2). We also used immunofluorescence microscopy and subcellular fractionation to determine the effects of insulin on subcellular localization of the predominant glucose transporter isoforms expressed in these cells.

## 6.3 Materials and methods

*Cell culture.* Clone 9 cells (ATCC, CRL-1439) were cultured in Kaighn's Modification of Ham's F-12 Medium containing 10% fetal bovine serum and were used between passage 10 and 20. H4IIE hepatoma cells (ATCC, CRL-1548) were cultured as previously described [10] and used between passage 5 and 20. L6 myoblasts (ATCC, CRL-1458) were cultured and differentiated to myotubes as previously described [11] and were used between passage 5 and 15. 3T3-L1 pre-adipocytes were cultured as previously described [12] and used between passage 10 and 15. All cells were maintained in 5% $CO_2$ at 37°C.

*RNA extraction and analyses.* Total RNA was extracted from cells using spin column-based RNA extraction kits (E.Z.N.A. Total RNA kits, Omega Bio-Tek, Norcross, GA). On-column DNase digestion was performed as specified by the manufacturer. RNA concentration and quality were assessed using the Experion™ RNA StdSens Analysis Kit (Bio-Rad Laboratories, Mississauga, ON) for the Experion™ electrophoresis system.

*Reverse transcription and quantitative PCR (qPCR).* One µg of RNA was reverse transcribed to complementary DNA using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., St. Bruno, QC, Canada) using the following program: 5 minutes at 25ºC; 30 minutes at 42°C; 5 minutes at 85°C. Primers for qPCR were

designed using Primer Blast software (NCBI, Bethesda, MD); sequences are presented in Table 6.1. Optimal annealing temperature and template quantity for each primer pair were determined by performing gradient qPCR and standard curve analyses. qPCR was carried out on a Mastercycler Gradient Realplex (Eppendorf, Hamburg, Germany) using the following program: 2 minutes at 95°C; 40 cycles of 15 seconds at 95°C, 15 seconds at the primer-specific annealing temperature, 20 seconds at 68°C. Melt curve analysis was used to determine specificity of primers for their intended product.

*SDS-PAGE and Western immunoblotting*. Total protein was extracted from cells using sample buffer containing 0.2 M Tris-HCl pH 6.8, 2% SDS and 30% glycerol. Lysates were sonicated and protein concentrations were determined using a colorimetric assay (BCA Protein Assay Kit, Pierce, Rockford, IL). SDS-PAGE and Western blotting were carried out as previously described [13]. Briefly, protein extracts (10 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 3% BSA-TBST before being probed with primary antibodies [GLUT1 (catalog no. 15309, Abcam, Cambridge, MA; catalog no. sc-7903, Santa Cruz Biotechnology, Santa Cruz, CA), GLUT3 (catalog no. sc-30107, Santa Cruz Biotechnology, Santa Cruz, CA), GLUT4 (catalog no. sc-1606, Santa Cruz Biotechnology, Santa Cruz CA), insulin receptor β, p42/44 ERK and eEF2 (Cell Signaling, Whitby, ON)]. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (diluted 1:10000 in 1% BSA-TBST). Immunoreactive bands were visualized by chemiluminescence (ECLPlus Western Blotting Detection System, Amersham Biosciences, Buckinghamshire, UK).

*$^{3}$H-2 deoxy-D-glucose uptake.* Cells were seeded into 24-well plates and maintained as described above. To assess the dose response effects of insulin on cellular glucose uptake, cells were treated with concentrations of insulin ranging from 0 to 1 µM, after which uptake of $^{3}$H-2 deoxy-D-glucose was conducted as previously described [14]. Briefly, cells were treated with insulin for 30 minutes, and then incubated with 1 µCi/ml

$^{3}$H-2 deoxy-D-glucose/0.1 mM 2 deoxy-D-glucose for 5 minutes. Glucose uptake was terminated by the addition of cold phloretin stop solution (0.3 mM phloretin in phosphate buffer), and cells were lysed in 0.1% SDS. An aliquot of cell lysate was transferred to a sample vial containing scintillation fluid, and the radioactivity of the samples was counted with an automatic liquid scintillation counter (Beckman LS-6500). The remaining cell lysate was assayed for protein as described above.

*Immunofluorescence*. Cells were fixed in 4% paraformaldehyde, permeabilized using 0.1% Triton X-100 in PBS (137 mM NaCl, 2 mM KCl, 10 mM $Na_2HPO_4$, 3 mM $KH_2PO_4$), blocked for 1 hour in 3% BSA-PBS and incubated overnight with primary antibody diluted 1:100 [GLUT1 (catalog no. sc-7903), GLUT3 (catalog no. sc-7582) and GLUT4 (catalog no. sc-1606), Santa Cruz Biotechnologies, Santa Cruz, CA]. Each staining experiment included a negative control that was incubated overnight with 3% BSA-PBS containing no primary antibody. After four washes with PBS, slides were then incubated with Alexa 488-conjugated secondary antibodies for 1 hour. After 4 consecutive 10 minute washes with 1x PBS, nuclei were stained with 5 µg/mL Hoechst 33342 (Sigma, St. Louis, MO). Fluorescent staining was visualized using an Olympus IX81F-3 microscope (Olympus, Richmond Hill, ON) and images were visualized with Infinity Analyze release 5.0.2 software (Lumenera Corporation, Nepean, ON).

*Subcellular fractionation.* Cells were seeded onto 150 mm plates and maintained as described above. On the day of experiments, cells were treated with 100 nM insulin for 10 minutes at 37°C. All subsequent steps were carried out on ice or at 4°C. Culture dishes were rinsed 3 times with 3 mL cold HES buffer [255 mM sucrose, 20 mM Hepes pH 7.4, 1 mM EDTA, 1x Halt™ protease inhibitor (Pierce, Rockford, IL)]. Cells were scraped into 3 mL of cold HES buffer and homogenized with twenty strokes in a Dounce homogenizer. The homogenate was centrifuged at 19 000 *g* for 20 minutes, and the resulting supernatant was further centrifuged at 40 000 *g* for 20 minutes to yield a pellet

designated as the high density microsomal fraction. The supernatant from the 40 000 *g* spin was centrifuged at 180 000 *g* for 75 minutes, yielding a pellet designated as the low density microsomal fraction. The pellet obtained from the initial 19 000 *g* spin was layered onto 1.12 M sucrose in HE buffer (20 mM Hepes pH7.4, 1 mM EDTA, 1x Halt™ protease inhibitor) and centrifuged at 100 000 *g* in a Beckman SW-41 Ti rotor for 60 minutes. This yielded a white fluffy band at the interface (plasma membrane). The interface containing the plasma membrane fraction was removed, resuspended in HES buffer and pelleted at 40 000 *g* for 20 minutes. All pellets were resuspended in sample buffer, and protein concentrations were quantified. Ten µg of the high density and low density fractions, and 20 µg of the plasma membrane fractions from untreated and insulin-treated cells were subjected to SDS-PAGE as described above.

*Data presentation and statistical analyses.* All quantitative data are presented as mean ± SEM. Differences in $^{3}$H-deoxy-D-glucose uptake among different insulin concentrations were assessed using one-way analysis of variance, with pre-planned contrasts to determine differences in means. Analyses were carried out using SAS version 9.3 statistical software (SAS Institute, Cary NC). Data are representative of three independent experiments.

## 6.4 Results

*Clone 9 cells express the GLUT3 gene.* Results from qPCR analyses of glucose transporter expression in commonly-used cell lines are presented in Table 6.2. In addition to the presence of GLUT1, Clone 9 cells expressed the highest level of GLUT3 of all the cell lines screened. Melt curve analysis of the qPCR products revealed only one product for all cell lines expressing GLUT3. To verify GLUT3-specific amplification, qPCR products were subjected to agarose gel electophoresis. Consistent with the results of melt curve analysis, a single band corresponding to the anticipated size of the

GLUT3 amplicon was observed (data not shown). The presence of the GLUT3 gene in Clone 9 cells was confirmed by conducting qPCR using a second set of primers directed against a different site within the GLUT3 gene, and consistent with results using the first primer, Clone 9 cells expressed the highest level of GLUT3 with only one PCR product of the anticipated size (data not shown). L6 rat skeletal muscle cells were the only other cell line that expressed the GLUT3 gene, and both undifferentiated myoblasts and differentiated myotubes contained relatively high levels of GLUT3 mRNA. Consistent with previous reports, Clone 9 cells also expressed the GLUT1 gene, but not the GLUT2 and GLUT4 genes. Interestingly, the gene for the fructose transporter, GLUT5, was also detected in Clone 9 cells. In H4IIE cells, GLUT1 and GLUT2 were the only glucose transporter genes expressed. The active sodium-dependent glucose transporters SGLT-1 and SGLT-2 were not detected in any of the cell lines screened.

*GLUT3 protein is present in Clone 9 cells.* To confirm the presence of GLUT3 and determine if this gene is translated in Clone 9 cells, we isolated protein from L6 myotubes, H4IIE and Clone 9 cells and used Western immunoblotting to determine the protein levels of GLUT1 and GLUT3 in these cells (Figure 6.1). Consistent with results at the gene level, GLUT3 protein was detected in Clone 9 cells, a finding confirmed with the use of two antibodies directed against different epitopes within the GLUT3 protein. Interestingly, Western blotting with two separate antibodies directed against different epitopes within the GLUT1 protein (one directed against the C-terminal domain and one directed against an internal sequence) demonstrated that, despite the presence of the GLUT1 gene, Clone 9 cells do not contain the GLUT1 protein. Similar to results at the gene level, GLUT1, but not GLUT3 protein, was detected in H4IIE cells.

*Insulin stimulates glucose uptake in Clone 9 liver cells.* In insulin-sensitive tissues such as adipose and skeletal muscle, rates of glucose uptake are enhanced by insulin-stimulated recruitment of GLUT4 from intracellular vesicles to the plasma membrane.

To determine the potential of insulin to enhance glucose uptake in Clone 9 cells, we first looked for expression of the insulin receptor in L6 myotubes, H4IIE and Clone 9 cells, and found this receptor was present in all three cell lines (Figure 6.2A). We then tested the concentration-dependent effect of insulin on 2-deoxy-D-glucose uptake, using L6 myotubes as a positive control. As anticipated, stimulation of L6 myotubes with insulin resulted in a concentration-dependent elevation in 2-deoxy-D-glucose uptake, with maximal uptake observed at 25 nM insulin (Figure 6.2B). Similarly, H4IIE cells also exhibited concentration-dependent elevations in 2-deoxy-D-glucose uptake that reached statistical significance at 250 nM insulin. Clone 9 cells also exhibited concentration-dependent elevations in 2-deoxy-D-glucose uptake, an effect that appeared unsaturated even at insulin concentrations as high as 1 µM (Figure 6.2C).

*Insulin promotes GLUT3 translocation to the plasma membrane in Clone 9 cells.*

Given the concentration-dependent stimulation of glucose uptake by insulin in Clone 9 cells, we sought to determine if insulin-stimulated translocation of GLUT3 to the plasma membrane might contribute to this effect. Immunofluorescence microscopy showed an even distribution of GLUT3 staining throughout the cytoplasm of untreated Clone 9 cells (Figure 6.3A, bottom panels). GLUT3 translocation to the plasma membrane, as evidenced by green staining localized around the periphery of the cells, could be detected after treatment with 10, 100 and 250 nM insulin. Similarly, H4IIE cells stained for GLUT1 after treatment with 10, 100 and 250 nM insulin displayed a redistribution of green staining from over the nucleus in untreated cells to the cytoplasm (Figure 6.3A, middle panels). Consistent with results from immunofluorescent staining, subcellular fractionation of Clone 9 cells treated with insulin showed a stronger immunoreactive band for GLUT3 in the plasma membrane fraction compared to untreated cells (Figure 6.3B, bottom panels). Similarly, insulin-treated H4IIE cells showed a stronger immunoreactive band for GLUT1 in the plasma membrane fraction compared to

untreated cells (Figure 6.3B, middle panels). As anticipated, plasma membrane fractions from L6 myotubes treated with insulin displayed higher plasma membrane levels of GLUT4 compared to untreated cells (Figure 6.3B, top panels)

**Table 6.1 Gene-specific primers used for qPCR analyses**

| Gene | Direction | Sequence |
|---|---|---|
| GLUT1 | Forward | 5'-TCC CAG CAG CTG TCG GGC AT-3' |
| | Reverse | 5'-CCA CGA CGA ACA GCG ACA CCA-3' |
| GLUT2 | Forward | 5'-TGT CTG TGT CCA GCT TTG CAG T-3' |
| | Reverse | 5'-TGC AGC CAA CAT GGC TTT GA-3' |
| GLUT3 | Forward | 5'-TCA ACC GCT TTG GCA GAC GCA-3' |
| | Reverse | 5'-AGG CGG CCC AGG ATC AGC AT-3' |
| GLUT4 | Forward | 5'-TCG GGG TGC CTT GGG AAC ACT-3' |
| | Reverse | 5'-ACC CAA CAC CTG GGC AAC CAG-3' |
| GLUT5 | Forward | 5'-TCA GCT CTT CAT CAC TGT GGG CAT-3' |
| | Reverse | 5'-TCT CAG GAA AGA AGG GCA GCA AGA-3' |
| SGLT-1 | Forward | 5'-TGC GGC TGA CAT ATC AGT CAT CGT-3' |
| | Reverse | 5'-TGA CCA CTT CCG ATG TTA CTG GCA-3' |
| SGLT-2 | Forward | 5'-CAT TGC CGC GTA TTT CCT GCT GG-3' |
| | Reverse | 5'-TGC CGA TGT TGC TGG CGA ACA-3' |

**Table 6.2 $C_t$ values for qPCR screening of glucose transporter expression in cell lines**

| | GLUT1 | GLUT2 | GLUT3 | GLUT4 | GLUT5 | SGLT-1 | SGLT-2 |
|---|---|---|---|---|---|---|---|
| **L6** | | | | | | | |
| **Myoblasts** | 24.2 ± 0.4 | N/D | 19.1 ± 0.5 | 24.3 ± 0.1 | 23.8 ± 0.1 | N/D | N/D |
| **Myotubes** | 26.6 ± 0.1 | N/D | 22.3 ± 0.7 | 25.5 ±0.01 | 26.1 ± 0.1 | N/D | N/D |
| **H4IIE** | 23.9 ± 1.9 | 25.3 ± 1.6 | N/D | N/D | N/D | N/D | N/D |
| **Clone 9** | 21.2 ± 0.3 | N/D | 18.5 ± 0.5 | N/D | 23.2 ± 0.1 | N/D | N/D |
| **3T3_L1** | | | | | | | |
| **Day 0** | 25.8 ± 0.7 | N/D | N/D | N/D | N/D | N/D | N/D |
| **Day 2** | 26.4 ± 0.4 | N/D | N/D | 28.0 ± 0.02 | N/D | N/D | N/D |
| **Day 4** | 28.2 ± 0.1 | N/D | N/D | 25.6 ± 0.01 | N/D | N/D | N/D |
| **Day 8** | 28.5 ± 0.1 | N/D | N/D | 24.2 ± 0.01 | N/D | N/D | N/D |

Data are presented as mean ± SEM (n=3). N/D =not detected.

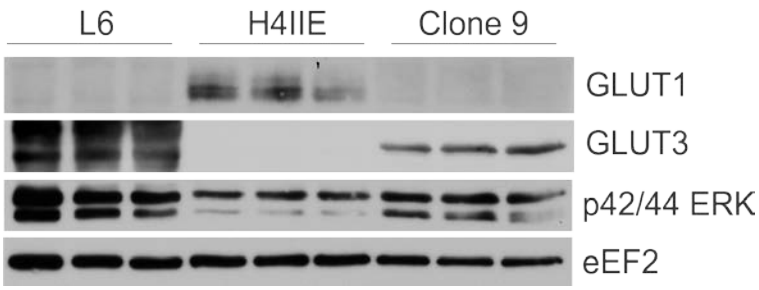


**Figure 6.1 The presence of GLUT3 protein in Clone 9 cells.** Protein lysates from H4IIE, Clone 9 and L6 myotubes were subjected to SDS-PAGE and Western immunoblotting with antibodies directed against GLUT1 and GLUT3. p42/44 ERK and eEF2 antibodies were used as loading controls for the replicates within each cell line. The GLUT1 blot represents the Western blot conducted using the primary antibody from Abcam (catalogue no. 15309). The GLUT3 blot represents the Western blot conducted using the primary antibody from Santa Cruz Biotechnology (catalogue no. sc-30107).

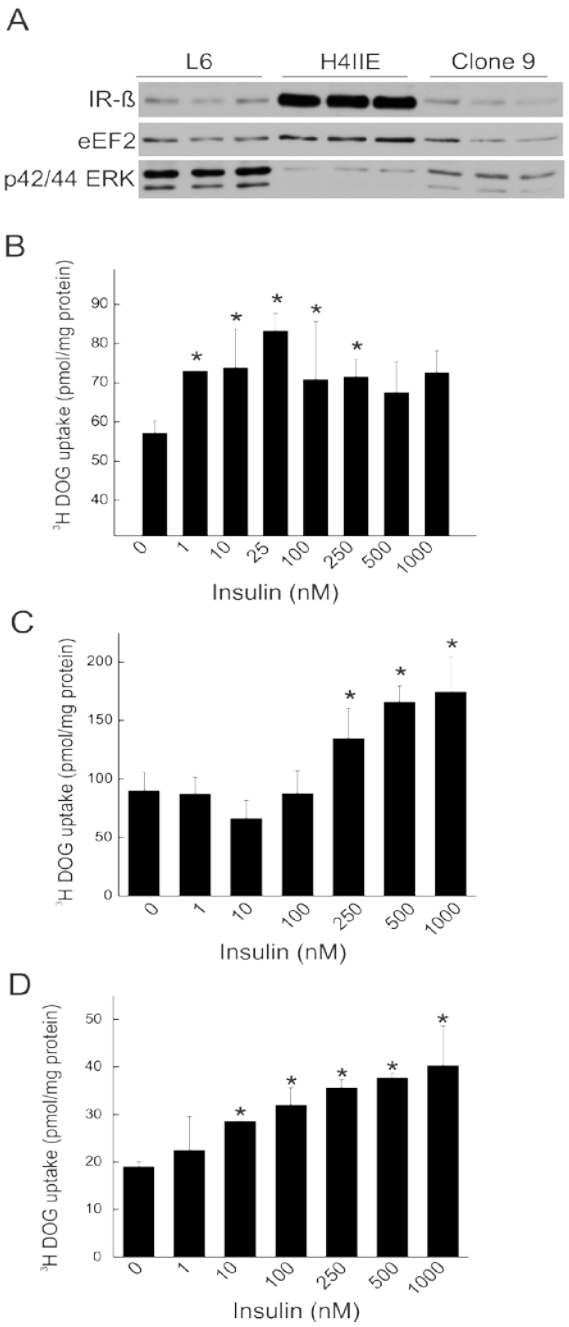


**Figure 6.2 Glucose uptake is sensitive to insulin in Clone 9 cells**. Western blot of insulin receptor β (IR-β) in L6 myotubes, H4IIE hepatomas and Clone 9 liver cells (A). p42/44 ERK and eEF2 antibodies were used as loading controls for the replicates within each cell line. Insulin dose-dependently enhanced glucose uptake in L6 myotubes (B), H4IIE hepatomas (C) and Clone 9 cells (D). Cells were stimulated with the indicated concentrations of insulin for 10 minutes before addition of $^3$H-deoxy-D-glucose ($^3$H DOG). Data are presented as means ± SEM. * denotes significantly different from 0 nM ($p<0.05$).

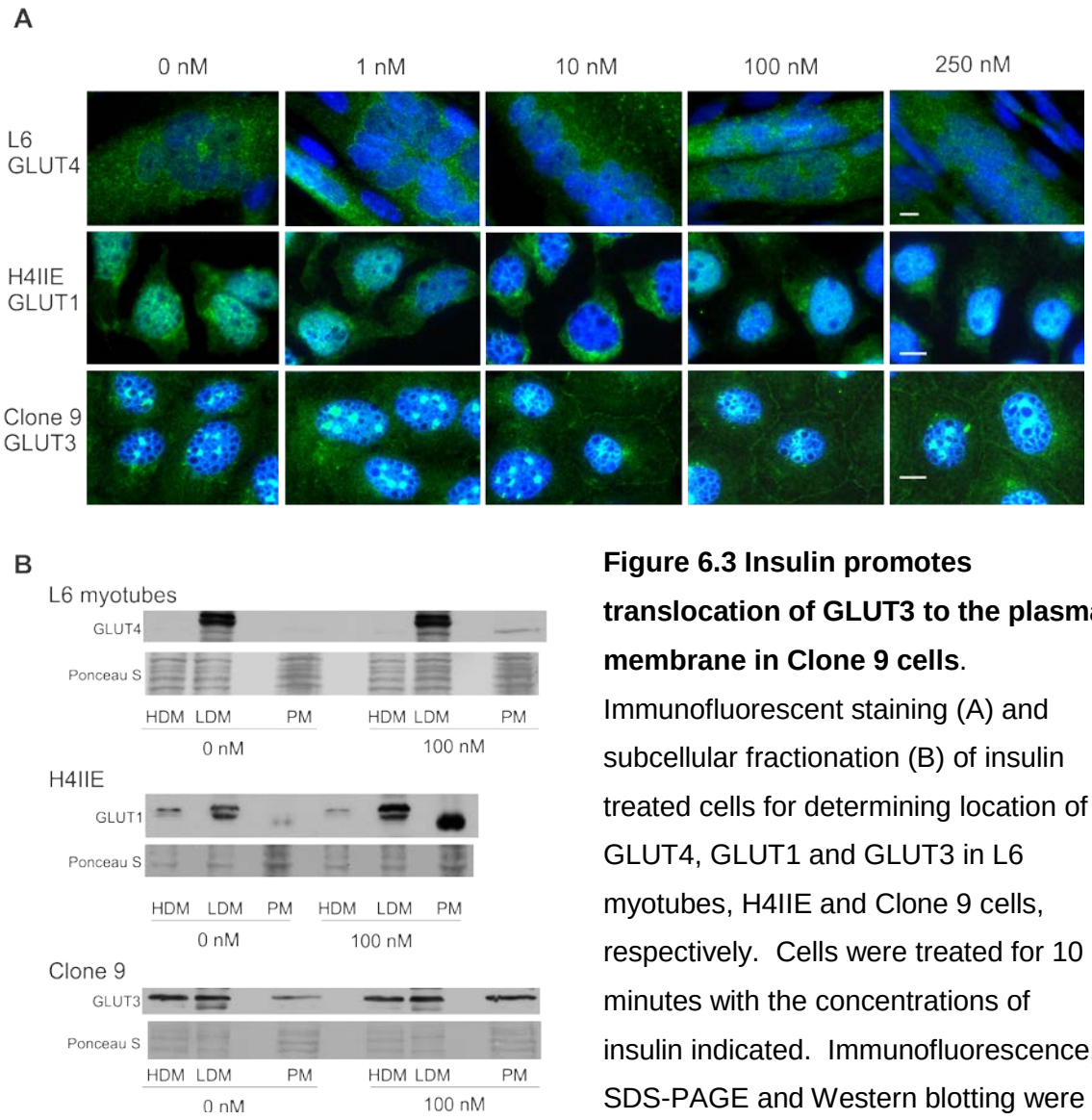


**Figure 6.3 Insulin promotes translocation of GLUT3 to the plasma membrane in Clone 9 cells**. Immunofluorescent staining (A) and subcellular fractionation (B) of insulin treated cells for determining location of GLUT4, GLUT1 and GLUT3 in L6 myotubes, H4IIE and Clone 9 cells, respectively. Cells were treated for 10 minutes with the concentrations of insulin indicated. Immunofluorescence, SDS-PAGE and Western blotting were carried out as described in Methods. For A, scale bar = 10 µm. For B, Ponceau S staining served as a loading control between fractions from untreated and insulin-treated cells. HDM = high density microsomal fraction; LDM = low density microsomal fraction; PM = plasma membrane.

## 6.5 Discussion

We have shown using several techniques that Clone 9 cells, originally reported to express only GLUT1, also express GLUT3 at both the gene and protein level. In addition, 2-deoxy-D-glucose uptake in these cells is up-regulated by insulin in a concentration-dependent manner, an observation likely related to higher levels of GLUT3 in the plasma membrane of cells treated with insulin. Detection of the GLUT3 gene and protein in Clone 9 cells contradicts numerous reports of exclusive expression of GLUT1 in this cell line [15,16,8]. Initial Northern blotting experiments demonstrated that Clone 9 cells express GLUT1 but not GLUT2 or GLUT4 [7]. However, GLUT3 was not included in this original study, and it is likely that absence of GLUT2 and GLUT4 mRNA was interpreted incorrectly to mean that GLUT1 is the only isoform expressed in this cell line. In an attempt to confirm exclusive expression of GLUT1 by qPCR, we discovered that Clone 9 cells also express GLUT3 and are the first to report its presence at both the gene and protein level. Furthermore, we have shown that Clone 9 cells do not express GLUT2, the predominant glucose transporter expressed in hepatocytes. Even though Clone 9 cells are untransformed and have not been immortalized through chemical or viral means most often associated with phenotypic changes, culture conditions may have been sufficient to induce a shift from predominant expression of GLUT2 to GLUT3, a phenomenon that has been documented in both primary pancreatic β-cells and hepatocytes [17,18]. As such, results from studies using Clone 9 cells to model hepatocyte glucose metabolism should be interpreted with caution.

Due to the perceived absence of other transporters, Clone 9 cells have been considered a good model in which to study GLUT1-mediated glucose uptake [15]. For instance, Clone 9 cells have been used to study the effects of azide, an inhibitor of oxidative phosphorylation, on rates of GLUT-1-mediated glucose uptake. Acute enhancement of glucose uptake in these cells after azide treatment occurs without

affecting GLUT1 mRNA or protein levels, suggesting involvement of post-translational mechanisms [19]. These post-translational mechanisms are likely not increases in plasma membrane GLUT1 content, but rather enhanced affinity of GLUT1 for glucose [5, 20]. Similarly, movement of GLUT1 to the plasma membrane is stimulated by thyroid hormone but not insulin in Clone 9 cells, despite increased glucose uptake with both of these hormones separately and a multiplicative enhancement with concurrent treatment [15]. Contrary to these previous reports, Western blot results using two commercial antibodies directed against different regions of the GLUT1 protein in the current study suggest that Clone 9 cells do not contain the GLUT1 protein. The reason for the discrepancy in GLUT1 detection between these commercial antibodies and the antibodies produced for and used in the above-mentioned studies is not known. If the absence of GLUT1 in Clone 9 cells is true, the presence of GLUT3 and its translocational control by insulin suggest that some of the properties of glucose metabolism previously attributed to GLUT1 may actually be the result of GLUT3 activity. Regardless, it is likely that elevated plasma membrane GLUT3 and thus GLUT3-mediated glucose uptake contributed to higher overall glucose uptake that we observed after insulin treatment. With the discovery of GLUT3 expression in Clone 9 cells, it may be worth determining if plasma membrane translocation of GLUT3 or increased affinity of GLUT3 for glucose contribute to the upregulation of glucose transport after azide or thyroid hormone treatment, as has been observed in osteoblasts [21].

Due to its relative abundance in the brain, GLUT3 is often considered the neuronal glucose transporter. Although neurons express the insulin receptor, treating neurons with insulin alone is insufficient to elicit increases in cellular glucose uptake [22,23]. Although insulin treatment does trigger GLUT3 translocation to the plasma membrane, it does not promote docking of GLUT3-containing vesicles to the plasma membrane in neurons; instead, vesicle fusion is dependent on membrane depolarization

[24]. As evidenced by results from our subcellular fractionation and immunofluorescence experiments with Clone 9 cells, insulin stimulation is sufficient to induce GLUT3 translocation to the plasma membrane, and likely also promotes GLUT3 vesicle fusion, as evidenced by elevated glucose uptake. Although we were able to show that insulin stimulates GLUT3 translocation in Clone 9 cells, the signaling pathways responsible for this movement are unknown. In L6 myotubes, GLUT3 translocation in response to insulin is regulated through an Akt-dependent pathway, much like that for GLUT4 [25]. In monocytes, GLUT3 translocation is regulated by IGF-1, which shares similar signaling pathways as insulin [26]. Further studies are required to elucidate the signaling pathways activated by insulin that regulate GLUT3 translocation in Clone 9 cells.

Although we have found that GLUT3 is expressed in Clone 9 cells, it does not discount the presence of other glucose transporters in this cell line. To date, 14 functional GLUT transporters have been identified [27,28]; thus, Clone 9 cells may express other transporters beyond those that were screened for in the current study.

In conclusion, expression of GLUT3 in Clone 9 cells and its subcellular redistribution contribute to insulin-stimulated elevations in glucose uptake observed in these cells. This information sheds new light on regulation of glucose metabolism in these cells, and may warrant a re-interpretation of results from previous studies claiming exclusive expression of GLUT1 in Clone 9 cells.

## 6.6 Literature Cited

[1] Hediger, M.A., Coady, M.J., Ikeda, T.S., and Wright, E.M. (1987). Expression cloning and cDNA sequencing of the Na+/glucose co-transporter. *Nature* 330, 379-81.

[2] Manolescu, A.R., Witkowska, K., Kinnaird, A., Cessford, T., and Cheeseman, C. (2007). Facilitated hexose transporters: new perspectives on form and function. *Physiology (Bethesda)* 22, 234-40.

[3] Weinstein, I.B., Orenstein, J.M., Gebert, R., Kaighn, M.E., and Stadler, U.C. (1975). Growth and structural properties of epithelial cell cultures established from normal rat liver and chemically induced hepatomas. *Cancer Res.* 35, 253-63.

[4] Behrooz, A. and Ismail-Beigi, F. (1998). Induction of GLUT1 mRNA in response to azide and inhibition of protein synthesis. *Mol. Cell. Biochem.* 187, 33-40.

[5] Shetty, M., Loeb, J.N., Vikstrom, K., and Ismail-Beigi, F. (1993). Rapid activation of GLUT-1 glucose transporter following inhibition of oxidative phosphorylation in clone 9 cells. *J. Biol. Chem.* 268, 17225-32.

[6] Kuruvilla, A.K., Perez, C., Ismail-Beigi, F., and Loeb, J.N. (1991). Regulation of glucose transport in Clone 9 cells by thyroid hormone. *Biochim. Biophys. Acta* 1094, 300-8.

[7] Hakimian, J. and Ismail-Beigi, F. (1991). Enhancement of glucose transport in clone 9 cells by exposure to alkaline pH: studies on potential mechanisms. *J. Membr. Biol.* 120, 29-39.

[8] Wei, Z., Peterson, J.M., and Wong, G.W. (2011). Metabolic regulation by C1q/TNF-related protein-13 (CTRP13): activation OF AMP-activated protein kinase and suppression of fatty acid-induced JNK signaling. *J. Biol. Chem.* 286, 15652-65.

[9] Simpson, I.A., Dwyer, D., Malide, D., Moley, K.H., Travis, A., and Vannucci, S.J. (2008). The facilitative glucose transporter GLUT3: 20 years of distinction. *Am. J. Physiol. Endocrinol. Metab.* 295, E242-53.

[10] Zahradka, P., Werner, J., and Yau, L. (1998). Expression and regulation of the insulin-like growth factor-1 receptor by growing and quiescent H4IIE hepatoma. *Biochim. Biophys. Acta* 1375, 131-9.

[11] Yau, L., Litchie, B., and Zahradka, P. (2004). MIBG, an inhibitor of arginine-dependent mono(ADP-ribosyl)ation, prevents differentiation of L6 skeletal myoblasts by inhibiting expression of myogenin and p21(cip1). *Exp. Cell Res.* 301, 320-30.

[12] Yeganeh, A., Stelmack, G.L., Fandrich, R., Halayko, A.J., Kardami, E., and and Zahradka, P. Connexin 43 phosphorylation and degradation are required for adipogenesis. *Biochim. Biophys. Acta* 1823, 1731-44.

[13] Yau, L., Elliot, T., Lalonde, C., and Zahradka, P. (1998). Repression of phosphoenolpyruvate carboxykinase gene activity by insulin is blocked by 3-aminobenzamide but not by PD128763, a selective inhibitor of poly(ADP-ribose) polymerase. *Eur. J. Biochem.* 253, 91-100.

[14] Mohankumar, S.K., Taylor, C.G., and Zahradka, P. (2012). Domain-dependent modulation of insulin-induced AS160 phosphorylation and glucose uptake by Ca2+/calmodulin-dependent protein kinase II in L6 myotubes. *Cell. Signal.* 24, 302-8.

[15] Shetty, M., Kuruvilla, A.K., Ismail-Beigi, F., and Loeb, J.N. (1996). Stimulation of glucose transport in Clone 9 cells by insulin and thyroid hormone: role of GLUT-1 activation. *Biochim. Biophys. Acta* 1314, 140-6.

[16] Hwang, D.Y. and Ismail-Beigi, F. (2002). Glucose uptake and lactate production in cells exposed to CoCl(2) and in cells overexpressing the Glut-1 glucose transporter. *Arch. Biochem. Biophys.* 399, 206-11.

[17] Tal, M., Thorens, B., Surana, M., Fleischer, N., Lodish, H.F., Hanahan, D., and Efrat, S. (1992). Glucose transporter isotypes switch in T-antigen-transformed pancreatic beta cells growing in culture and in mice. *Mol. Cell. Biol.* 12, 422-32.

[18] Rhoads, D.B., Takano, M., Gattoni-Celli, S., Chen, C.C., and Isselbacher, K.J. (1988). Evidence for expression of the facilitated glucose transporter in rat hepatocytes. *Proc. Natl. Acad. Sci. U. S. A.* 85, 9042-6.

[19] Shetty, M., Loeb, J.N., and Ismail-Beigi, F. (1992). Enhancement of glucose transport in response to inhibition of oxidative metabolism: pre- and posttranslational mechanisms. *Am. J. Physiol.* 262, C527-32.

[20] Shi, Y., Liu, H., Vanderburg, G., Samuel, S.J., Ismail-Beigi, F., and Jung, C.Y. (1995). Modulation of GLUT1 intrinsic activity in clone 9 cells by inhibition of oxidative phosphorylation. *J. Biol. Chem.* 270, 21772-8.

[21] Zoidis, E., Ghirlanda-Keller, C., and Schmid, C. (2012). Triiodothyronine stimulates glucose transport in bone cells. *Endocrine* 41, 501-11.

[22] Schulingkamp, R.J., Pagano, T.C., Hung, D., and Raffa, R.B. (2000). Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci. Biobehav. Rev.* 24, 855-72.

[23] Heidenrich, K.A., Gilmore, P.R., and Garvey, W.T. (1989). Glucose transport in primary cultured neurons. *J. Neurosci. Res.* 22, 397-407.

[24] Uemura, E. and Greenlee, H.W. (2006). Insulin regulates neuronal glucose uptake by promoting translocation of glucose transporter GLUT3. *Exp. Neurol.* 198, 48-53.

[25] Hajduch, E., Alessi, D.R., Hemmings, B.A., and Hundal, H.S. (1998). Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. *Diabetes* 47, 1006-13.

[26] Dimitriadis, G., Maratou, E., Boutati, E., Kollias, A., Tsegka, K., Alevizaki, M., Peppa, M., Raptis, S.A., and Hadjidakis, D.J. (2008). IGF-I increases the recruitment of GLUT4 and GLUT3 glucose transporters on cell surface in hyperthyroidism. *Eur. J. Endocrinol.* 158, 361-6.

[27] Joost, H.G., Bell, G.I., Best, J.D., Birnbaum, M.J., Charron, M.J., Chen, Y.T., Doege, H., James, D.E., Lodish, H.F., Moley, K.H., Moley, J.F., Mueckler, M., Rogers, S., Schurmann, A., Seino, S., and Thorens, B. (2002). Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. *Am. J. Physiol. Endocrinol. Metab.* 282, E974-6.

[28] Uldry, M. and Thorens, B. (2004). The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch.* 447, 480-9.

# Chapter 7: Consumption of buckwheat modulates the post-prandial response of selected gastrointestinal satiety hormones in individuals with type 2 diabetes mellitus

Danielle M. Stringer[1,3], Carla G.Taylor[1,2,3], Paulyn Appah[4], Heather J. Blewett[2,3], Peter Zahradka[1,2,3]

[1]Departments of [1]Human Nutritional Sciences and [2]Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

[3]Canadian Centre for Agri-food Research in Health and Medicine, St. Boniface Research Centre, Winnipeg, Manitoba, Canada

[4]Food Development Centre, Portage La Prairie, Manitoba, Canada

## 7.1 Abstract

The purpose of the current study was two-fold: (1) to compare the acute 3-hour post-prandial response of glucose, insulin and other gastrointestinal hormones known to influence food intake and glucose metabolism after consumption of a food product made from whole grain buckwheat flour versus rice flour and (2) to determine the effect of daily consumption of a food product made from whole grain buckwheat flour on fasting glucose, lipids and apolipoproteins. Healthy participants or those with type 2 diabetes mellitus (T2DM) consumed either buckwheat or rice crackers. Blood samples were collected at baseline and 15, 30, 45, 60, 120 and 180 minutes after consumption. In a second phase of the study, participants consumed one serving of buckwheat crackers daily for 1 week; fasting blood samples from day 1 and day 7 were analyzed. Post-prandial plasma glucagon-like peptide-1, glucose-dependent insulinotropic peptide and pancreatic polypeptide were altered after consuming buckwheat versus rice crackers. Interestingly, changes in these hormones did not lead to changes in post-prandial glucose, insulin or C-peptide concentrations. Significant correlations were observed between both fasting concentrations and post-prandial responses of several of the hormones examined. Interestingly, certain correlations were present only in the healthy participant group or the T2DM group. There was no effect of consuming buckwheat for one week on fasting glucose, lipids or apolipoproteins in either the healthy participants or those with T2DM. Although the buckwheat cracker did not modify acute glycemia or insulinemia, it was sufficient to modulate gastrointestinal satiety hormones.

## 7.2 Introduction

Diabetes mellitus is a significant global public health concern. Despite available pharmacological treatments, the majority of patients with type 2 diabetes mellitus (T2DM) do not meet recommendations for glycemic control, exposing them to risks of uncontrolled hyperglycemia [1]. Modest reductions in weight can improve glycemic control and cardiovascular risk factors in patients with established T2DM [2,3]. Thus, the identification of food products that aid in glycemic management, potentially through reduction of energy intake and weight, may complement traditional pharmacological therapies.

Buckwheat is a broad-leafed herbaceous annual plant that is often classified and treated as a cereal crop. Epidemiological evidence from China has shown a significantly lower prevalence of hyperglycemia in a community consuming buckwheat as a staple food compared to a community that subsisted primarily on rice, suggesting a potential role for buckwheat in glycemic control [4]. Similarly, intervention studies using baked products prepared with buckwheat flour have demonstrated that consuming buckwheat daily for as little as 30 days reduces fasting blood glucose concentrations in individuals with diabetes [5,6]. Unfortunately, it is difficult to evaluate critically the potential of buckwheat as a dietary tool for diabetes management, as these previous studies do not specify the type and dose of buckwheat used. Previously, we have observed significant reductions in fasting and fed state serum glucose concentrations in a rat model for type 1 diabetes given an intragastric dose of a buckwheat extract devoid of soluble fibre [7], an effect that was also observed in a rodent model for T2DM [8]. Buckwheat is a source of several phytochemicals that may positively influence glucose metabolism, [9-11]; therefore, the presence of these compounds may provide a mechanistic link between consumption of buckwheat and improved glycemic control. Overall, while evidence

suggests consuming buckwheat may attenuate hyperglycemia, controlled studies to support such a claim are lacking.

The objective of the current study was to determine the efficacy of a food product made from buckwheat to modify glucose metabolism in both healthy people and those with T2DM. Glucose metabolism is influenced by the actions of several hormones secreted from the gastrointestinal tract that also act as satiety signals; thus we also sought to determine how consumption of buckwheat modulates the post-prandial response of several of these hormones. To determine if potential beneficial effects of consuming buckwheat were due to improvements in acute versus longer term glucose control, the study was separated into acute and chronic phases.

## 7.3 Materials and Methods

*Participants.* This was a single-blind, controlled, cross-over study conducted in Winnipeg, Manitoba, Canada. Sample size was based on previous studies of other food products reporting acute glucose-lowering effects. Volunteers were males and females aged 18 to 70 years, not taking medications to manage glycemia and not having acute or chronic conditions affecting glycemic control, nutrient absorption or secretion of gastrointestinal hormones (e.g. inflammatory bowel disease, gastrointestinal resection or surgery, etc.). Participants in the control (CTL) group were generally healthy with $HbA_{1c}$ <6% as determined at screening. Participants in the T2DM group had relatively well-controlled T2DM managed by diet alone, with $HbA_{1c}$ <7.5% as determined at screening. Participants were asked to maintain their current lifestyle and level of physical activity, and to refrain from consuming foods containing buckwheat.

This study was conducted according to the guidelines in the Declaration of Helsinki and all procedures were approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Participants

provided written informed consent consistent with guidelines for the protection of human research participants.

*Dietary Interventions.* Crackers were chosen as the food product to deliver the buckwheat. Recipe formulation and production methods were developed by the Food Development Centre in Portage La Prairie, Manitoba. Whole grain flour was milled from dehulled buckwheat (*Fagopyrum esculentum* moenech, variety Koto) and used to produce the buckwheat crackers. The reference product chosen for this study was a similar cracker made from commercially available rice flour. Portion sizes of both cracker products contained 50 grams of available carbohydrate. Results of nutrient analysis are presented in Table 7.1.

*Sample collection.* Acute phase**.** Participants attended two sessions (scheduled at least one week apart) where they consumed one portion of either the buckwheat or rice crackers, in random order. Following an overnight fast, participants came to the clinic and open venous access was secured. A fasting blood sample was obtained, after which the participant consumed their randomly assigned product. Additional blood samples were collected 15, 30, 60, 120 and 180 minutes after the first bite. Blood was collected in EDTA-containing vacutainer tubes, and dipeptidyl peptidase IV inhibitor (Millipore, St. Charles, MO, USA) and Halt™ protease inhibitor (Pierce, Rockford, IL, USA) were added immediately before centrifugation (1800 *g*,10 minutes, 4°C) to obtain plasma.

Chronic phase**.** Fasting blood samples were collected the morning of day 0 (baseline), day 2, day 4 and day 7. Blood was processed in the same manner as the acute phase.

*Analytic Procedures.* Acute phase**.** Plasma glucose concentrations were determined using an enzymatic colourimetric kit (Genzyme Diagnostics, Charlottetown, PE, Canada). Concentrations of insulin, C-peptide, glucose-dependent insulinotropic peptide (GIP), pancreatic polypeptide (PP) and active ghrelin were determined using a Human

Metabolic Hormone Milliplex kit (Millipore). The intra- and inter-assay precision and lower limit of detection for each analyte were: insulin – 3%, 10%, 8.97 pmol/L; C-peptide – 4%, 8%, 7.67 pmol/L; GIP – 4%, 6%, 0.1 pmol/L; PP – 2%, 5%, 0.68 pmol/L; active ghrelin – 3%, 9%, 0.76 pmol/L. Active glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) concentrations were determined using an electrochemiluminescent immunoassay (Meso Scale Discovery, Gaithersburg, MD). The lower limit of detection for GLP-1 was 0.07 pmol/L and for PYY was 9.25 pmol/L; intra- and inter-assay precision for these assays was not provided by the manufacturer. Chronic phase**.** Chronic phase samples were assayed for glucose as described above. Total, LDL and HDL cholesterol, triglycerides, alanine aminotransferase and aspartate aminotransferase were quantified in plasma using a clinical auto analyzer (cobas c111 analyzer, Roche Diagnostics, Laval, QC, Canada). Apoplipoprotein AI, B and CIII concentrations were quantified using a Human Apolipoprotein Milliplex kit (Millipore). The intra- and inter-assay precision for each analyte was: apolipoprotein AI – 5%, 11%; apolipoprotein B – 8%, 14%; apolipoprotein CIII – 5%, 13%. Compliance during the chronic phase was assessed through self-reported intakes recorded in study diaries and short interviews conducted during clinic visits.

*Data analysis and statistics.* Demographic and clinical variables are presented as mean ± SD. Biochemical and experimental values are presented as mean ± SEM. Overall glucose and satiety hormone responses were calculated as the incremental AUC (iAUC) determined from all blood sampling time points during the 3 hour acute phase visits. Data that were not normally distributed were log transformed before statistical analysis. Differences among groups at blood sampling time points were determined using a mixed model repeated measures ANOVA, with participants as a random effect and treatment and diabetes as fixed effects. Differences in iAUC measurements were assessed using 2-way ANOVA to determine effects of treatment or diabetes, as well as an interaction

between treatment and diabetes. Differences among specific groups were identified using pre-planned comparisons of least squared means.

Insulin resistance was assessed by calculating HOMA-IR [12]. Correlations between HOMA-IR, fasting concentrations of hormones and iAUC for hormones were assessed using Spearman's correlation coefficient.

For chronic phase results, repeated measures ANOVA with 2 factor analysis was used to determine the effect of time (visit number) and diabetes (CTL or T2DM), as well as significant interactions between time and diabetes. Differences among groups were determined using pre-planned comparisons of least squared means.

Data were analyzed with SAS version 9.2 software (SAS Institute, Inc., Cary, NC, USA). The significance level was $p \leq 0.05$ for main effects and means testing, and $p \leq 0.10$ for interactions (to reduce the risk of missing interactions). Sample sizes are specified in each figure legend.

## 7.4 Results

*Participants.* Demographic and clinical characteristics of participants are presented in Table 7.2. Participants with T2DM were older, weighed more, had a higher BMI and HbA1c than healthy participants.

*Plasma measurements.* Acute phase. Results for glucose, insulin and C-peptide are presented in Figure 7.1 and 7.2. Analyses of plasma glucose values at blood sampling time points revealed a significant effect of time ($p<0.0001$), diabetes ($p<0.0001$) and a significant interaction between time and diabetes ($p=0.0012$), due to the fact that blood glucose concentrations of the T2DM groups peaked at a later time and took longer to return to baseline (Figure 7.1 A). For insulin analysis, sample size of the CTL group was reduced from 12 to 11 due to consistent results below the lower limit of detection for the assay in one participant. Significant effects of time ($p<0.0001$) and diabetes ($p<0.0001$),

and a significant interaction between time and diabetes (p=0.0004) were detected; specifically, peak insulin concentrations occurred later in participants with T2DM and failed to return to baseline concentrations (Figure 7.1 B). Similarly, significant effects of time (p<0.0001) and diabetes (p<0.0001), and a significant interaction between time and diabetes (p<0.0001) were also detected for plasma C-peptide, with the peak C-peptide concentration of participants with T2DM occurring later in the test (Figure 7.1 C). A significant effect of diabetes was detected in iAUC for plasma glucose, insulin and C-peptide (Figures 7.2 A, B and C), with the T2DM group having higher iAUCs. Consuming buckwheat compared to rice crackers did not affect plasma concentrations of glucose, insulin or C-peptide.

Comparing GLP-1 concentrations at each time point, only a significant effect of time (p<0.001) was detected (Figure 7.3 A). iAUC for GLP-1 revealed a trend (p=0.07) towards higher iAUC after consumption of the buckwheat crackers compared to the rice crackers (Figure 7.4 A). There was a significant interaction (p=0.08) between group and treatment (Figure 7.4 A), stemming from a higher iAUC for GLP-1 in the control participants after consumption of buckwheat compared to rice crackers. In contrast, there was a significant interaction (p=0.0008) between time and treatment for plasma GIP after consumption of rice; specifically, plasma GIP concentrations were higher after 60 minutes in participants that consumed rice crackers compared to those that consumed buckwheat crackers (Figure 7.3 B). This contributed to a significant effect of treatment on iAUC for GIP (p=0.004), with the iAUC for GIP being lower after consumption of buckwheat compared to rice crackers (Figure 7.4 B).

Analyses of individual time points revealed that participants with T2DM started with significantly lower fasting plasma ghrelin concentrations, and maintained lower concentrations compared to the control participants throughout the remainder of the test (Figure 7.5 A). Despite this, participants with T2DM still retained a typical post-prandial

ghrelin response (i.e. plasma concentrations of ghrelin decreased after consumption of a food product). Participants with T2DM had a significantly lower iAUC for active ghrelin than control participants, but consumption of buckwheat crackers did not affect iAUC for ghrelin when compared to the rice crackers (Figure 7.6 A).

Repeated measures analysis of plasma PP concentrations revealed a significant interaction between time and diabetes ($p<0.001$; Figure 7.5 B). Both groups that consumed buckwheat crackers had higher post-prandial PP concentrations compared to groups consuming rice crackers, but this difference was only significant between the CTL buckwheat and CTL rice at t60. There was a trend ($p=0.06$) for iAUC of PP to be higher after consumption of the buckwheat crackers compared to the rice crackers (Figure 7.6 B). For PYY analysis, sample size was reduced to 6 for the control groups and 7 for the T2DM groups because values of PYY for many participants at several time points fell below the lower limit of detection for the PYY assay. There was no significant effect of diabetes or treatment on PYY concentrations at specific time points or on iAUC for PYY (Figure 7.5 C and 7.6 C).

Correlations between fasting hormone concentrations are presented in Table 7.3. There was a strong positive association between PYY and GLP-1 in all participants, a relationship remaining in both groups when analyzed separately. Positive associations were also observed between GIP and each of PYY, PP, insulin, and GLP-1, stemming from relationships between these hormones in the participants with T2DM only. Conversely, overall positive associations observed between PYY and insulin, and insulin and GLP-1, could be attributed to the relationships between these hormones in the control participants only. Interestingly, the positive association between PYY and PP was only observed in participants with T2DM. Overall, ghrelin was inversely associated with GIP only.

Correlations between iAUC for hormones are presented in Table 7.4. Of note, there was a strong inverse relationship between iAUC for ghrelin and insulin, and a strong positive relationship between iAUC for PYY and GLP-1, and PYY and PP, but after consumption of buckwheat crackers only. Conversely, the strong inverse relationship between iAUC for PYY and GIP was observed only after consumption of rice crackers. Interestingly, there was no overall association between iAUC for PYY and PP, but when separated by group, these two hormones were inversely associated in the control participants and a positively associated in the T2DM participants. Consistent with the fasting values, the positive overall association between iAUC for insulin and GLP-1 was observed only in the control participants. Despite no overall relationship, positive associations were found between PP and GLP-1, insulin and GIP, and GLP-1 and ghrelin in the T2DM participants only.

Although the inverse correlation between fasting insulin and fasting ghrelin concentrations was minor (-0.272) and displayed only a trend towards significance ($p=0.078$), inverse correlations were observed between HOMA-IR and fasting ghrelin (-0.590, $p=0.006$) as well as HOMA-IR and iAUC for ghrelin (-0.540, $p=0.004$). No associations existed between HOMA-IR and fasting concentrations or iAUC for the other hormones measured.

Chronic phase**.** All participants completed the chronic phase and there were no adverse effects reported. Although samples from all time points during the chronic phase were analyzed, no significant effects of time and no interactions between time and diabetes were detected, and thus only the values from day 0 and day 7 are reported (Table 7.5). Fasting plasma glucose was significantly higher ($p=0.0002$) in the participants with T2DM compared to the control group, and there was no effect of consuming buckwheat crackers daily for one week on fasting plasma glucose in either of the two groups.

Patients with T2DM are at an increased risk of cardiovascular disease and its associated morbidities and mortality; thus, we examined the effects of daily consumption of buckwheat on plasma markers of cardiovascular disease as well as plasma apolipoproteins apoAI, apo B, apoCIII, and the ratio between apo B and apo AI. Participants with T2DM had a trend ($p=0.08$) for lower HDL cholesterol compared to the healthy participants, but LDL and total cholesterol were not different between the two groups. Consistent with lower HDL cholesterol, apo AI (the major apolipoprotein on HDL particles) was lower ($p=0.02$) in the participants with T2DM compared to the control participants. There were no significant differences in apo B or apo CIII between the two groups. Furthermore, there was no effect of consuming buckwheat crackers for 1 week on plasma lipids, apolipoproteins or the ratio of apo B to Apo AI.

There was no effect of consuming buckwheat crackers for one week on plasma concentrations of ALT and AST, liver enzymes used to assess safety.

**Table 7.1 Nutrient content of buckwheat and rice crackers**

| *Nutrient (per serving size)* | *Rice (66 g)[3]* | *Buckwheat (76 g)[3]* |
|---|---|---|
| Energy[1] (kCal) | 296.3 | 340.5 |
| Calories from fat[1] (kCal) | 73.2 | 95.0 |
| Carbohydrates[1] (g) | 51.8 | 53.1 |
| Protein[2] (g) | 4.0 | 10.7 |
| Saturated fat[2] (g) | 0.9 | 1.0 |
| Monounsaturated fat[2] (g) | 5.0 | 6.4 |
| Polyunsaturated fat[2] (g) | 2.2 | 3.2 |
| Omega 3 fatty acids[2] (g) | 0.5 | 0.7 |
| Omega 6 fatty acids[2] (g) | 1.7 | 2.5 |
| Trans fatty acids[2] (g) | <0.1 | <0.1 |
| Total fat[2] (g) | 8.1 | 10.6 |
| Sodium[2] (mg) | 291.0 | 364.0 |
| Calcium[2] (mg) | 9.5 | 17.3 |
| Iron[2] (mg) | 0.2 | 3.5 |
| Potassium[2] (mg) | 63.6 | 298.6 |
| Moisture[2] (g) | 1.1 | 2.2 |
| Ash[2] (g) | 0.9 | 1.9 |
| Cholesterol (mg) | <1 | <1 |
| Vitamin C (mg) | <1 | <1 |
| Vitamin A[2] (IU) | <10 | <10 |
| Total dietary fibre (g) | 2.0 | 3.2 |

[1]based on Atwater method
[2] based on AOAC method
[3]serving sizes based on amount required to achieve 50 grams of available carbohydrate
< denotes none detected, followed by the detection limit
Analysis performed by SGS Canada Inc. (Vancouver, BC, Canada).

**Table 7.2 Participant demographic and clinical characteristics**

| | ***CTL*** | ***T2DM*** |
|---|---|---|
| Male/Female (n) | 6/6 | 5/7 |
| Age (y) | 37.3 ± 16.3 | 60.8 ± 6.7 |
| Body weight (kg) | 72.5 ± 17.3 | 95.2 ± 17.1 |
| BMI ($kg/m^2$) | 23.5 ± 3.4 | 32.4 ± 6.6 |
| HbA1c (%) | 5.2 ± 0.2 | 6.5 ± 0.4 |
| Duration of T2DM (y) | *N/A* | 4.12 ± 3.71 |

Data are presented as mean ± SD.

**Table 7.3 Correlations between fasting hormones measured during the acute phase**

| | Ghrelin | GIP | GLP-1 | Insulin | PP |
|---|---|---|---|---|---|
| **PYY** | -0.238 (0.242) | -0.066 (0.749) | **0.612 (0.001)** | **0.590 (0.001)** | -0.022 (0.914) |
| CTL | 0.333 (0.112) | 0.107 (0.620) | **0.449 (0.028)** | **0.580 (0.003)** | 0.245 (0.248) |
| T2DM | 0.148 (0.490) | **0.423 (0.040)** | **0.709 (0.0001)** | -0.260 (0.220) | **0.493 (0.014)** |
| **PP** | 0.106 (0.499) | **0.345 (0.016)** | 0.157 (0.285) | **0.590 (0.001)** | |
| CTL | 0.225 (0.290) | 0.069 (0.748) | -0.013 (0.952) | 0.207 (0.333) | |
| T2DM | 0.240 (0.258) | **0.527 (0.008)** | 0.344 (0.100) | 0.259 (0.222) | |
| **Insulin** | -0.272 (0.078) | **0.287 (0.048)** | **0.361 (0.012)** | | |
| CTL | 0.208 (0.329) | -0.011 (0.958) | **0.518 (0.010)** | | |
| T2DM | 0.189 (0.376) | **0.451 (0.027)** | 0.174 (0.415) | | |
| **GLP-1** | -0.262 (0.090) | **0.356 (0.013)** | | | |
| CTL | -0.259 (0.222) | 0.101 (0.637) | | | |
| T2DM | 0.212 (0.321) | **0.595 (0.002)** | | | |
| **GIP** | **-0.315 (0.039)** | | | | |
| CTL | -0.327 (0.119) | | | | |
| T2DM | 0.271 (0.201) | | | | |

Results are presented as Spearman's rho correlation coefficient with p-values in parentheses (insulin: overall n=46; n=23 for the CTL group and n=24 for the T2DM group; GLP-1, GIP and ghrelin: overall n= 48; n=24 for both groups; PP: overall n=46; n=24 for the CTL group and n=23 for the T2DM group; PYY: overall n=26; n=12 for the CTL group and n=14 for the T2DM group). Bolded values indicate significant correlations.

**Table 7.4 Correlations between iAUC for hormones during the acute phase**

| | Ghrelin | GIP | GLP-1 | Insulin | PP |
|---|---|---|---|---|---|
| **PYY** | -0.086 (0.702) | -0.331 (0.098) | 0.249 (0.221) | -0.101 (0.624) | 0.208 (0.309) |
| *Rice* | 0.231 (0.447) | **-0.599 (0.031)** | 0.093 (0.762) | -0.126 (0.681) | 0.165 (0.590) |
| *BW* | 0.041 (0.850) | -0.155 (0.471) | **0.611 (0.002)** | 0.201 (0.345) | **0.455 (0.026)** |
| *CTL* | -0.321 (0.263) | -0.451 (0.106) | 0.123 (0.675) | 0.407 (0.149) | **-0.574 (0.032)** |
| *T2DM* | 0.139 (0.518) | -0.042 (0.846) | **0.654 (0.001)** | 0.208 (0.330) | **0.565 (0.004)** |
| **PP** | -0.017 (0.919) | 0.222 (0.130) | **0.290 (0.046)** | -0.181 (0.252) | |
| *Rice* | 0.300 (0.155) | 0.259 (0.223) | 0.326 (0.120) | 0.024 (0.910) | |
| *BW* | -0.050 (0.816) | 0.262 (0.216) | 0.218 (0.305) | 0.147 (0.492) | |
| *CTL* | -0.022 (0.917) | 0.050 (0.818) | 0.072 (0.738) | 0.088 (0.683) | |
| *T2DM* | 0.170 (0.427) | 0.304 (0.148) | **0.468(0.021)** | 0.201 (0.347) | |
| **Insulin** | **-0.519 (0.001)** | 0.038 (0.813) | 0.011 (0.946) | | |
| *Rice* | **-**0.074 (0.730) | 0.388 (0.061) | 0.344 (0.100) | | |
| *BW* | 0.059 (0.783) | -0.018 (0.932) | 0.290 (0.169) | | |
| *CTL* | -0.104 (0.629) | 0.095 (0.658) | **0.536 (0.007)** | | |
| *T2DM* | 0.176 (0.411) | **0.429 (0.036)** | 0.062 (0.773) | | |
| **GLP-1** | 0.040 (0.810) | 0.174 (0.237) | | | |
| *Rice* | 0.220 (0.301) | 0.249 (0.240) | | | |
| *BW* | 0.245 (0.248) | 0.034 (0.875) | | | |
| *CTL* | -0.029 (0.893) | 0.094 (0.663) | | | |
| *T2DM* | **0.530 (0.008)** | 0.235 (0.268) | | | |
| **GIP** | -0.243 (0.142) | | | | |
| *Rice* | -0.008 (0.971) | | | | |
| *BW* | -0.241 (0.256) | | | | |
| *CTL* | -0.233 (0.274) | | | | |
| *T2DM* | 0.069 (0.749) | | | | |

Data are presented as Spearman's rho correlation coefficient with p-values in parentheses (insulin: overall n=46; n=23 for rice, BW, CTL and T2DM); GLP-1, GIP and ghrelin: overall n=48; n= 24 for rice, BW, CTL and T2DM; PP: overall n=46; n=23 for rice, BW, CTL and T2DM; PYY: overall n=26; n=13 for rice, BW, CTL and T2DM). Bolded values indicate significant correlations. BW= buckwheat, CTL=healthy participants, T2DM = participants with T2DM.

**Table 7.5 Chronic phase clinical measurements of participants**

| | *Control* | | *T2DM* | | *p-value* | | |
|---|---|---|---|---|---|---|---|
| | ***Day 0*** | ***Day 7*** | ***Day 0*** | ***Day 7*** | ***Time*** | ***Diabetes*** | ***T*D†*** |
| **Glucose (mmol/L)** | 4.70 ± 0.09 | 4.53 ± 0.16 | 6.89 ± 0.37 | 6.98 ± 0.37 | 0.8702 | 0.0002 | 0.9081 |
| **Cholesterol** | | | | | | | |
| Total *(mmol/L)* | 4.71 ± 0.45 | 4.60 ± 0.42 | 4.27 ± 0.24 | 4.39 ± 0.22 | 0.1525 | 0.6279 | 0.1181 |
| LDL *(mmol/L)* | 2.91 ± 0.36 | 2.84 ± 0.32 | 2.65 ± 0.21 | 2.75 ± 0.17 | 0.8402 | 0.8460 | 0.2722 |
| HDL *(mmol/L)* | 1.51 ± 0.13 | 1.53 ± 0.13 | 1.25 ± 0.11 | 1.24 ± 0.11 | 0.4413 | 0.0880 | 0.5160 |
| **Apolipoproteins** | | | | | | | |
| ApoAI *(g/L)* | 2.08 ± 0.14 | 1.97 ± 0.53 | 1.51 ± 0.16 | 1.85 ± 0.17 | 0.1263 | 0.0221 | 0.8413 |
| ApoB *(g/L)* | 0.083 ± 0.014 | 0.055 ± 0.008 | 0.074 ± 0.016 | 0.069 ± 0.013 | 0.2520 | 0.1488 | 0.2917 |
| ApoCIII *(g/L)* | 0.227 ± 0.038 | 0.193 ± 0.047 | 0.136 ± 0.022 | 0.159 ± 0.018 | 0.2792 | 0.1351 | 0.3209 |
| ApoB:ApoAI | 0.039 ± 0.004 | 0.037 ± 0.004 | 0.035 ± 0.003 | 0.042 ± 0.005 | 0.7886 | 0.7163 | 0.2123 |
| **Triglycerides (mmol/L)** | 1.26 ± 0.26 | 1.25 ± 0.30 | 1.27 ± 0.13 | 1.45 ± 0.15 | 0.8236 | 0.2503 | 0.0793 |
| **Liver enzymes** | | | | | | | |
| ALT *(U/L)* | 14.6 ± 1.2 | 14.5 ± 1.1 | 21.6 ± 2.4 | 24.1 ± 2.6 | 0.5350 | 0.0058 | 0.0947 |
| AST *(U/L)* | 18.9 ± 1.4 | 18.3 ± 1.1 | 20.5 ± 2.0 | 21.1 ± 1.7 | 0.9517 | 0.3597 | 0.5430 |

Data are presented as mean ± SEM (n=12). † = time*diabetes interaction.

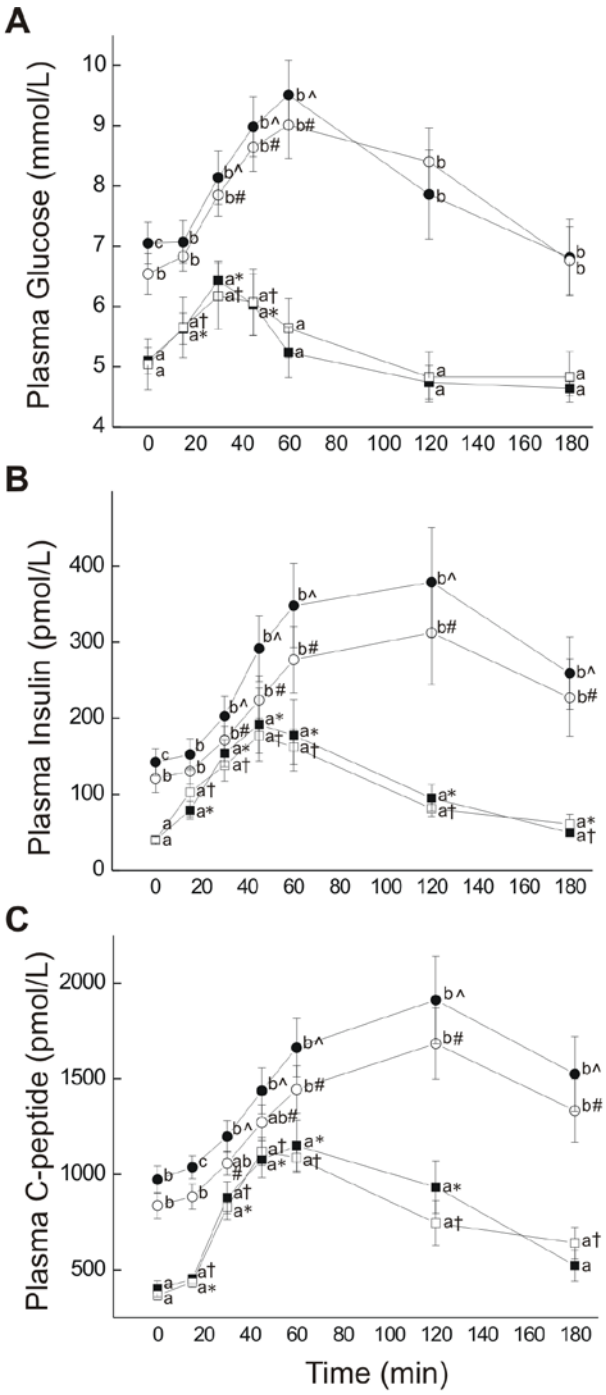


**Figure 7.1 Plasma response of glucose (A), insulin (B) and C-peptide (C) after consumption of rice crackers or buckwheat crackers in control participants and those with T2DM.** Repeated measures ANOVA of log transformed values showed a significant effect of time ($p<0.0001$), diabetes ($p<0.0001$) and a significant time*diabetes interaction ($p=0.001$, $p=0.004$ and $p<0.0001$ for glucose, insulin and C-peptide, respectively). Black squares represent control participants consuming rice crackers, white squares represent control participants consuming buckwheat crackers, black circles represent participants with T2DM consuming rice crackers, white circles represent participants with T2DM consuming buckwheat crackers. Means within each time point with different letters are significantly different ($p<0.05$). * denotes significantly different from t=0 in control participants consuming rice crackers, † denotes significantly different from t=0 in control participants consuming buckwheat crackers, ^ denotes significantly different from t=0 in T2DM participants consuming rice crackers, and # denotes significantly different from t=0 in T2DM participants consuming buckwheat crackers ($p<0.05$). T2DM = type 2 diabetes mellitus.

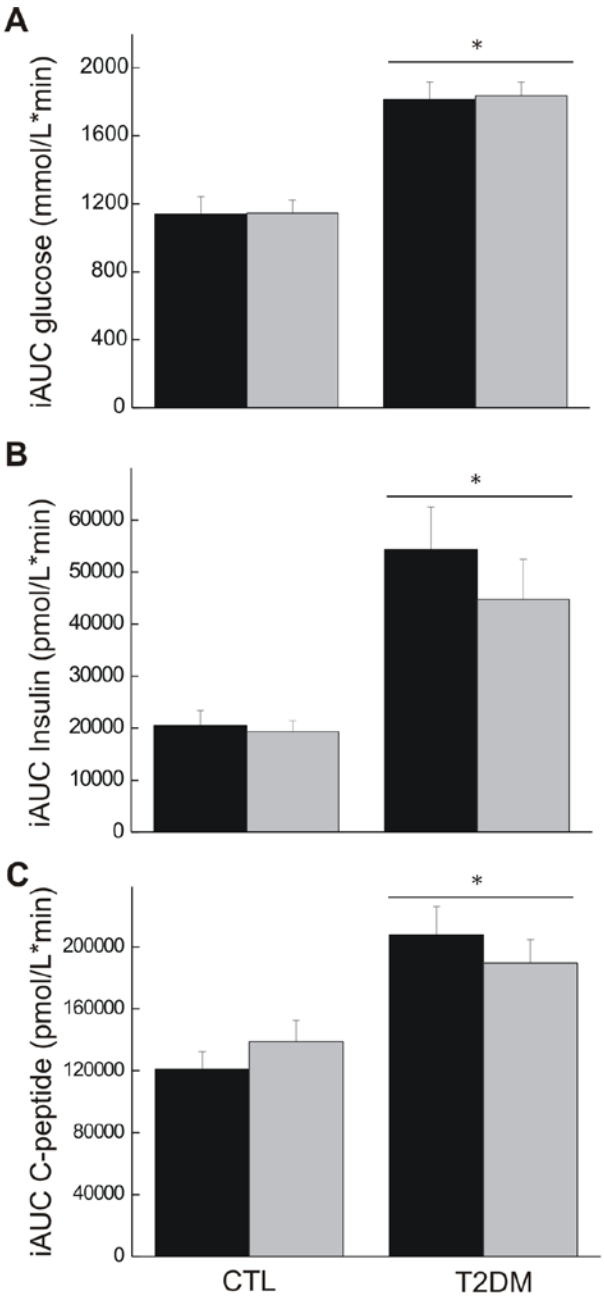


**Figure 7.2 iAUC for glucose (A), insulin (B) and C-peptide (C) calculated from concentrations of each analyte during the 3-hour postprandial test.** Two-way ANOVA of untransformed (C-peptide) and log transformed data (glucose and insulin) showed a significant effect of diabetes for each analyte ($p<0.0001$, $p=0.0006$ and $p=0.007$ for glucose, insulin and C-peptide, respectively). Black bars represent participants consuming rice crackers, grey bars represent participants consuming buckwheat crackers. * denotes significantly different from control groups ($p<0.05$). Data are presented as untransformed mean ± SEM with n=12 for all groups for glucose and C-peptide, n=11 for the control groups and n=12 for the T2DM groups for insulin. iAUC = incremental area under the curve, CTL = control, T2DM = type 2 diabetes mellitus.

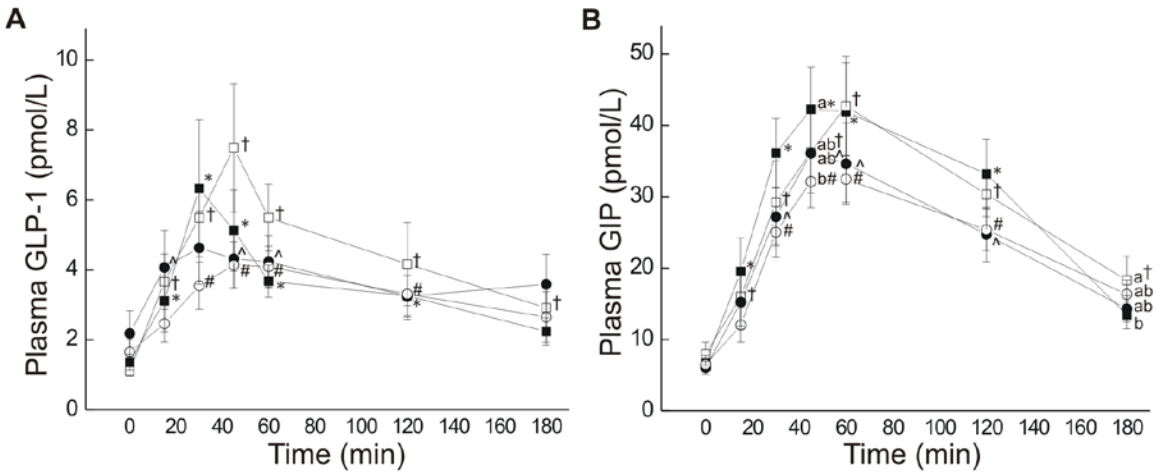


**Figure 7.3 Plasma response of GLP-1 (A) and GIP (B) after consumption of rice crackers or buckwheat crackers in control participants and those with T2DM.** For GLP-1, only a significant effect of time ($p<0.0001$) was detected. Repeated measures ANOVA of GIP values showed a significant effect of time ($p<0.0001$), treatment ($p<0.0039$) and a significant interaction between time and treatment ($p<0.0008$). Black squares represent control participants consuming rice crackers, white squares represent control participants consuming buckwheat crackers, black circles represent participants with T2DM consuming rice crackers, white circles represent participants with T2DM consuming buckwheat crackers. * denotes significantly different from t=0 in control participants consuming rice crackers, † denotes significantly different from t=0 in control participants consuming buckwheat crackers, ^ denotes significantly different from t=0 in T2DM participants consuming rice crackers, and # denotes significantly different from t=0 in T2DM participants consuming buckwheat crackers ($p<0.05$). For GIP, means within each time point with different letters are significantly different ($p<0.05$). GLP-1 = glucagon-like peptide-1, GIP = glucose-dependent insulinotropic peptide, T2DM =type 2 diabetes.

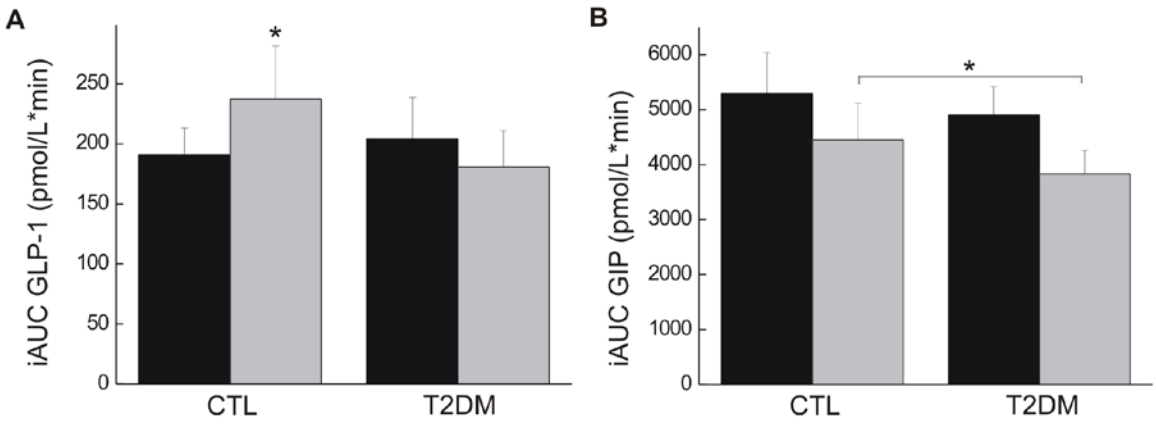


**Figure 7.4 iAUC for GLP-1 (A) and GIP (B) calculated from concentrations of each hormone during the 3-hour post-prandial test.** Two-way ANOVA of iAUC for GLP-1 showed a trend towards an effect of treatment (p=0.07) and a significant interaction between diabetes and treatment (p=0.08; *denotes significantly higher than control participants consuming buckwheat crackers). For iAUC for GIP, there was a significant effect of treatment (p=0.004; *denotes significantly different from groups consuming rice crackers). Black bars represent participants consuming rice crackers, grey bars represent participants consuming buckwheat crackers. Data are presented as untransformed mean ± SEM with n=12 for all groups. iAUC = incremental area under the curve; GLP-1 = glucagon-like peptide-1, GIP = glucose-dependent insulinotropic peptide, CTL = control, T2DM = type 2 diabetes mellitus.

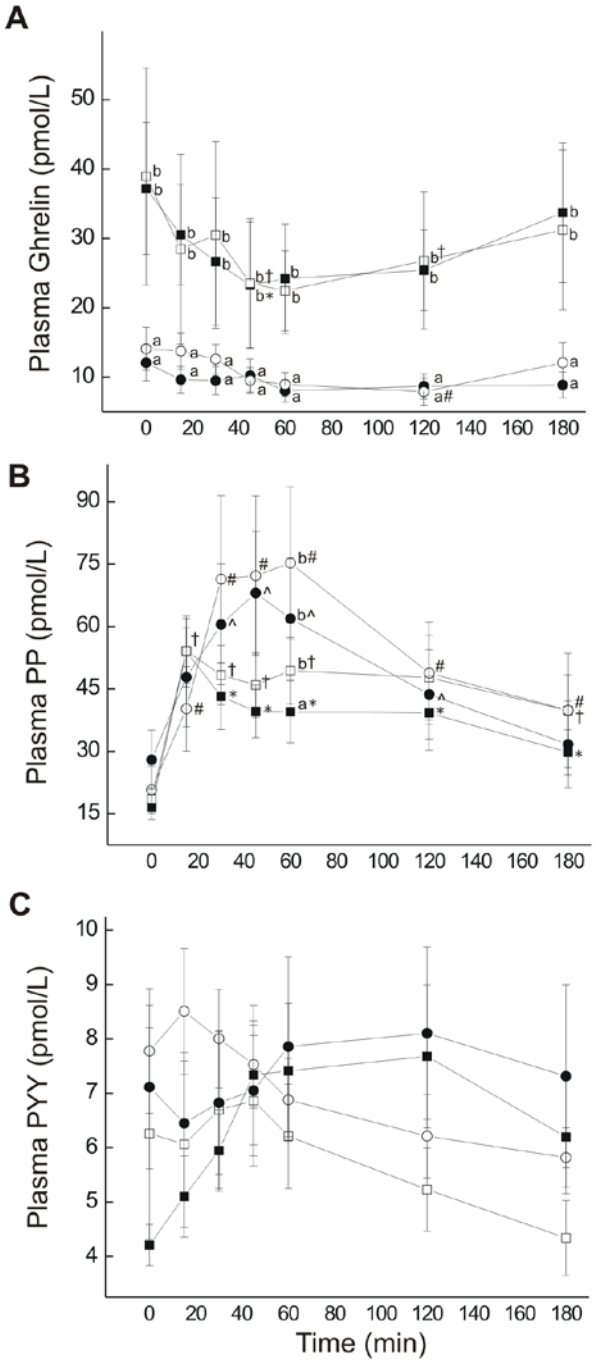


**Figure 7.5 Plasma response of ghrelin (A), PP (B) and PYY (C) after consumption of rice crackers or buckwheat crackers in control participants and those with T2DM.** Repeated measures ANOVA of ghrelin concentrations at individual time points of the test showed significant effects of time ($p=0.01$) and diabetes ($p=0.01$; means within time points with different letters are significantly different). Repeated measures ANOVA of log transformed PP concentrations at individual time points showed a significant effect of time ($p<0.001$), as well as a significant interaction between time and diabetes ($p<0.001$). Repeated measures ANOVA of log transformed PYY concentrations at individual time points showed a significant effect of time ($p=0.004$). Black squares represent control participants consuming rice crackers, white squares represent control participants consuming buckwheat crackers, black circles represent participants with T2DM consuming rice crackers, white circles represent participants with T2DM consuming buckwheat crackers. * denotes significantly different from t=0 in control participants consuming rice crackers, † denotes significantly different from t=0 in control participants consuming buckwheat crackers, ^ denotes significantly different from t=0 in T2DM participants consuming rice crackers, and # denotes significantly different from t=0 in T2DM participants consuming buckwheat crackers ($p<0.05$). PP = pancreatic polypeptide, PYY = peptide YY, T2DM = type 2 diabetes mellitus.

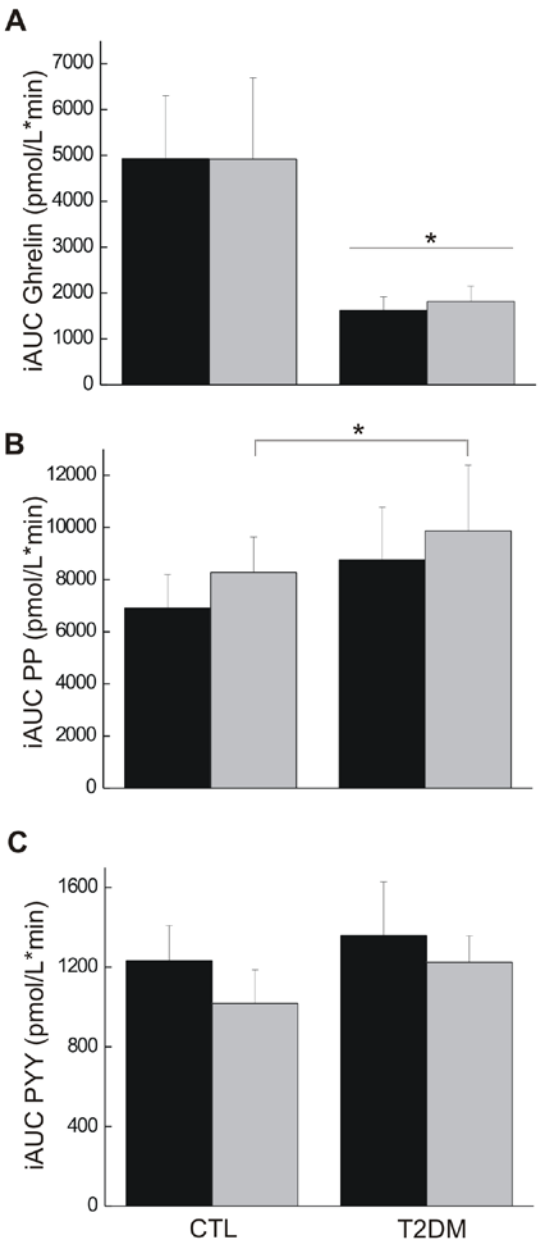


**Figure 7.6 iAUC for ghrelin (A), PP (B) and PYY (C) calculated from concentrations of each analyte during the 3-hour post-prandial test.** Two-way ANOVA of log transformed data showed a significant effect of diabetes (p=0.008; * denotes significantly lower than control participants) for ghrelin, a significant effect of treatment (p=0.063; * denotes significantly higher than groups consuming rice crackers) for PP, but no significant effects for PYY. Black bars represent groups consuming rice crackers, grey bars represent groups consuming buckwheat crackers. Data are presented as untransformed mean ± SEM with n=12 for all groups for ghrelin, n=12 for the Control groups and n=11 for the T2DM groups for PP, and n=6 for the control groups and n=7 for the T2DM groups for PYY. iAUC=incremental area under the curve, PP=pancreatic polypeptide, PYY=peptide YY, CTL=control, T2DM=type 2 diabetes mellitus.

## 7.5 Discussion

Results from the current study demonstrate that consumption of a serving of buckwheat containing 50 grams of available carbohydrate differentially modulates the acute plasma response of several gastrointestinal hormones, including GLP-1 and GIP. While we observed higher post-prandial GLP-1 response after consumption of buckwheat, the opposite effect was seen with the post-prandial GIP response. As incretin hormones, GLP-1 and GIP exert their short-term effects on post-prandial glucose metabolism by increasing insulin secretion from pancreatic β-cells [13]. However, despite differential modulation of incretin hormones by buckwheat, post-prandial glucose and insulin responses remained unaffected. This divergent response of two hormones with similar effects on insulin secretion and glucose metabolism is puzzling, but may explain the lack of effect on post-prandial glucose and insulin responses between the rice and buckwheat crackers, as any insulinotropic or glucose-lowering effects of GLP-1 stemming from consuming buckwheat might have been negated by a reduction in GIP secretion.

Clinical trials are exploring pharmacological activation of the signaling systems of several anorexigenic hormones, such as PYY and PP, for reduction in caloric intake as anti-obesity therapy [14,15]. Modifying the post-prandial responses of these hormones using foods has been examined largely in the context of food products varying in amount or type of dietary fibre. For example, 10.5 grams of insoluble wheat bran fibre incorporated into a bread product significantly elevated the post-prandial response of PYY [16]. Likewise, a mixture of inulin and oligofructose can increase post-prandial PP concentrations [17], while consumption of extruded breakfast cereals supplemented with β-glucan is associated with higher post-prandial cholecystokinin concentrations [18]. Changes in post-prandial satiety signals elicited by foods are not limited to changes in anorexigenic hormones: psyllium and inulin fibres have been shown to modify secretion

of the orexigenic hormone ghrelin [19,20]. In the current study, consumption of buckwheat was associated with higher post-prandial concentrations of PP and GLP-1 (which also has anorexigenic effects in addition to incretin effects), suggesting that food products made with buckwheat may promote satiety. However, we did not observe changes in post-prandial concentrations of PYY or active ghrelin. These hormones are secreted in proportion to macronutrient composition and energy content of meals [21], [22]; therefore, the lack of difference in the post-prandial response of these two hormones between the two cracker products may be explained by their similar macronutrient and caloric contents.

Results from epidemiological and animal studies suggest that buckwheat may positively influence glucose metabolism and hyperglycemia [4-6]; thus, we hypothesized that a food product made from buckwheat would favourably modify acute glucose metabolism in both healthy people and those with T2DM. However, we did not observe changes in acute post-prandial glucose or insulin after buckwheat consumption; furthermore, consumption of buckwheat for 1 week did not reduce fasting plasma glucose, total, LDL and HDL cholesterol, apolipoproteins or triglyceride concentrations. Results from previous studies have found anti-hyperglycemic actions of a buckwheat extract devoid of fibre in animal models for both T1DM and T2DM [7,8], an effect which may occur independent of the insulin-mimetic actions of inositol phosphoglycans or flavanoids [23]. Regardless of the identity of the compound in the extract used in the animal studies and its mode of action, no effect was seen in post-prandial glucose concentrations in the current study with whole-grain buckwheat. These contrasting results may be explained by an insufficient dose of the anti-hyperglycemic molecule in the serving size of our cracker product.

The identity of the active component in buckwheat responsible for the higher levels of satiety hormones that were observed in the current study remains unknown. Soluble fibres, such as oligofructose can augment gastrointestinal hormone secretion and feelings of fullness in an overweight population [24]. Short-chain fatty acids produced from fermentation of oligofructose increase expression of GLP-1 and GIP in intestinal endocrine cells [25,26]; however, it is unknown if these short-chain fatty acid products also stimulate the release of these hormones from intracellular stores. Although a considerable portion of the dietary fibre found in buckwheat is of the soluble variety, little information is available on the specific types of soluble fibre. Buckwheat contains several non-starch polysaccharides consisting of xylose, mannose, galactose and glucuronic acid [27], and it is possible that short-chain fatty acids produced from fermentation of these non-starch polysaccharides stimulated secretion of several gastrointestinal satiety hormones. However, this remains a hypothesis until a direct link between products of intestinal fermentation and secretion of gastrointestinal hormones from enteroendocrine cells is established.

Consistent with previous reports [28,29], our participants with T2DM had significantly lower ghrelin concentrations throughout the acute test compared to controls; furthermore, strong inverse correlations were observed between post-prandial insulin and ghrelin, and between insulin resistance and the post-prandial ghrelin response. The degree of insulin resistance in our participants, as assessed using HOMA-IR, was not associated with post-prandial responses of other gastrointestinal satiety hormones. Interestingly, when separated by treatment, the inverse relationship between HOMA-IR and the post-prandial ghrelin response was only observed when participants consumed rice crackers, suggesting that consumption of rice crackers impeded feelings of fullness as the degree of insulin resistance increased.

We found several correlations between fasting concentrations and the post-prandial responses of gastrointestinal hormones, a novel finding given that several of these relationships were observed only in participants with T2DM. For instance, the positive associations observed between fasting concentrations of GIP and each of PP, PYY and GLP-1 were only observed in participants with T2DM. Conversely, positive associations between insulin and GLP-1, and PYY and insulin were observed only in healthy participants, an observation consistent with the blunted incretin response in T2DM [13]. Although relationships between insulin and several gastrointestinal hormones have been studied previously, little information is available regarding relationships among gastrointestinal hormones and interactions between their secretion. We found significant relationships between the post-prandial responses of several gastrointestinal hormones that were influenced by consumption of buckwheat but not rice (PYY and GLP-1; PYY and PP). In the case of PYY and GLP-1, which are secreted from the same enteroendocrine cell, this may indicate the presence of a compound in buckwheat that stimulates the secretory activity of these cells. Given the trend towards higher iAUC for PP after consumption of buckwheat, the positive association between iAUC for PYY and PP observed in participants consuming buckwheat may signify a relationship between PYY and PP secretion stimulated by a bioactive in buckwheat. Future cell culture studies may help to clarify these relationships.

There are several strengths of the current study. This is the first single-blinded, randomized, reference product-controlled trial examining the effect of consuming a food product made with buckwheat on the acute response of glucose and other gastrointestinal satiety hormones. Inclusion of healthy participants and those with T2DM allowed us to assess how T2DM could influence potential effects of buckwheat consumption and relationships between fasting and post-prandial gastrointestinal

hormones. In addition, recruiting participants with diet-managed T2DM allowed us to eliminate potentially confounding effects of anti-diabetic medications.

A limitation of the current study is that we did not conduct subjective assessments of satiety in our participants and therefore we cannot conclude that buckwheat consumption leads to stronger feelings of fullness and thus reduced food intake. It is difficult to link quantitative changes in satiety hormones to degree of reduced food intake, and changes in post-prandial responses of satiety hormones do not always correlate with changes in perceived satiety [30]. Regardless, higher plasma concentrations of satiety hormones after consumption of buckwheat is encouraging and warrants further studies.

In conclusion, consumption of a food product made from whole-grain buckwheat flour containing 50 grams of available carbohydrate differentially affected post-prandial responses of GLP-1 and GIP but did not lead to changes in post-prandial glucose or insulin in healthy participants or those with T2DM. Post-prandial levels of several gastrointestinal satiety hormones were higher after consuming buckwheat, suggesting greater satiety. If the proposed effects on increased satiety and reduced caloric intake hold true in the longer term, it is possible that consumption of foods made with buckwheat may promote reduced energy intake, an important consideration for those attempting to control glycemia.

## 7.6 Literature Cited

[1] American Diabetes Association. (2012). Executive summary: Standards of medical care in diabetes--2010. *Diabetes Care* 35 Suppl 1, S4-10.

[2] Wing, R.R., Lang, W., Wadden, T.A., Safford, M., Knowler, W.C., Bertoni, A.G., Hill, J.O., Brancati, F.L., Peters, A., Wagenknecht, L., and the Look AHEAD Research Group. (2011). Benefits of Modest Weight Loss in Improving Cardiovascular Risk Factors in Overweight and Obese Individuals With Type 2 Diabetes. *Diabetes Care* 34, 1481-6.

[3] Look AHEAD Research Group, Pi-Sunyer, X., Blackburn, G., Brancati, F.L., Bray, G.A., Bright, R., Clark, J.M., Curtis, J.M., Espeland, M.A., Foreyt, J.P., Graves, K., Haffner, S.M., Harrison, B., Hill, J.O., Horton, E.S., Jakicic, J., Jeffery, R.W., Johnson, K.C., Kahn, S., Kelley, D.E., Kitabchi, A.E., Knowler, W.C., Lewis, C.E., Maschak-Carey, B.J., Montgomery, B., Nathan, D.M., Patricio, J., Peters, A., Redmon, J.B., Reeves, R.S., Ryan, D.H., Safford, M., Van Dorsten, B., Wadden, T.A., Wagenknecht, L., Wesche-Thobaben, J., Wing, R.R., and Yanovski, S.Z. (2007). Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial. *Diabetes Care* 30, 1374-83.

[4] Zhang, H.W., Zhang, Y.H., Lu, M.J., Tong, W.J., and Cao, G.W. (2007). Comparison of hypertension, dyslipidaemia and hyperglycaemia between buckwheat seed-consuming and non-consuming Mongolian-Chinese populations in Inner Mongolia, *China. Clin. Exp. Pharmacol. Physiol*. 34, 838-44.

[5] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia and

hyperlipidemia. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, pp 458-64.

[6] Wang J, Liu Z, Fu X, Run M (1992) A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, 465-7.

[7] Kawa, J.M., Taylor, C.G., and Przybylski, R. (2003). Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. *J. Agric. Food Chem*. 51, 7287-91.

[8] Yao, Y., Shan, F., Bian, J., Chen, F., Wang, M., and Ren, G. (2008). D-chiro-inositol-enriched tartary buckwheat bran extract lowers the blood glucose level in KK-Ay mice. *J. Agric. Food Chem*. 56, 10027-31.

[9] Kwon, O., Eck, P., Chen, S., Corpe, C.P., Lee, J.H., Kruhlak, M., and Levine, M. (2007). Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J*. 21, 366-77.

[10] Johnston, K., Sharp, P., Clifford, M., and Morgan, L. (2005). Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett*. 579, 1653-7.

[11] Fonteles, M.C., Almeida, M.Q., and Larner, J. (2000). Antihyperglycemic effects of 3-O-methyl-D-chiro-inositol and D-chiro-inositol associated with manganese in streptozotocin diabetic rats. *Horm. Metab. Res*. 32, 129-32.

[12] Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., and Turner, R.C. (1985). Homeostasis model assessment: insulin resistance and beta-cell

function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28, 412-9.

[13] Gautier, J.F., Choukem, S.P., and Girard, J. (2008). Physiology of incretins (GIP and GLP-1) and abnormalities in type 2 diabetes. *Diabetes Metab*. 34 Suppl 2, S65-72.

[14] Halford, J.C., Boyland, E.J., Blundell, J.E., Kirkham, T.C., and Harrold, J.A. (2010). Pharmacological management of appetite expression in obesity. *Nat. Rev. Endocrinol*. 6, 255-69.

[15] Hussain, S.S. and Bloom, S.R. (2011). The pharmacological treatment and management of obesity. *Postgrad. Med*. 123, 34-44.

[16] Weickert, M.O., Spranger, J., Holst, J.J., Otto, B., Koebnick, C., Mohlig, M., and Pfeiffer, A.F. (2006). Wheat-fibre-induced changes of postprandial peptide YY and ghrelin responses are not associated with acute alterations of satiety. *Br. J. Nutr*. 96, 795-8.

[17] Cani, P.D., Lecourt, E., Dewulf, E.M., Sohet, F.M., Pachikian, B.D., Naslain, D., De Backer, F., Neyrinck, A.M., and Delzenne, N.M. (2009). Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *Am. J. Clin. Nutr. 90*, 1236-43.

[18] Beck, E.J., Tosh, S.M., Batterham, M.J., Tapsell, L.C., and Huang, X.F. (2009). Oat beta-glucan increases postprandial cholecystokinin levels, decreases insulin response and extends subjective satiety in overweight subjects. *Mol. Nutr. Food Res*. 53, 1343-51.

[19] Tarini, J. and Wolever, T.M. (2010). The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Appl. Physiol. Nutr. Metab*. 35, 9-16.

[20] Karhunen, L.J., Juvonen, K.R., Flander, S.M., Liukkonen, K.H., Lahteenmaki, L., Siloaho, M., Laaksonen, D.E., Herzig, K.H., Uusitupa, M.I., and Poutanen, K.S. (2010). A psyllium fiber-enriched meal strongly attenuates postprandial gastrointestinal peptide release in healthy young adults. *J. Nutr*. 140, 737-44.

[21] El Khoury, D. and Hwalla, N. (2010). Metabolic and appetite hormone responses of hyperinsulinemic normoglycemic males to meals with varied macronutrient compositions. *Ann. Nutr. Metab*. 57, 59-67.

[22] Adrian, T.E., Ferri, G.L., Bacarese-Hamilton, A.J., Fuessl, H.S., Polak, J.M., and Bloom, S.R. (1985). Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89, 1070-7.

[23] Curran, J.M., Stringer, D.M., Wright, B., Taylor, C.G., Przybylski, R., and Zahradka, P. (2010). Biological response of hepatomas to an extract of Fagopyrum esculentum M. (buckwheat) is not mediated by inositols or rutin. *J. Agric. Food Chem*. 58, 3197-204.

[24] Parnell, J.A. and Reimer, R.A. (2009). Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults. *Am. J. Clin. Nutr*. 89, 1751-9.

[25] Reimer, R.A., Thomson, A.B., Rajotte, R.V., Basu, T.K., Ooraikul, B., and McBurney, M.I. (1997). A physiological level of rhubarb fiber increases proglucagon gene expression and modulates intestinal glucose uptake in rats. *J. Nutr*. 127, 1923-8.

[26] Reimer, R.A. and McBurney, M.I. (1996). Dietary fiber modulates intestinal proglucagon messenger ribonucleic acid and postprandial secretion of glucagon-like peptide-1 and insulin in rats. *Endocrinology* 137, 3948-56.

[27] The Canadian Special Crops Association. (2008). Buckwheat-Information. http://www.specialcrops.mb.ca/crops/buckwheat.html. Accessed September 25th, 2012.

[28] Poykko, S.M., Kellokoski, E., Horkko, S., Kauma, H., Kesaniemi, Y.A., and Ukkola, O. (2003). Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes* 52, 2546-53.

[29] McLaughlin, T., Abbasi, F., Lamendola, C., Frayo, R.S., and Cummings, D.E. (2004). Plasma ghrelin concentrations are decreased in insulin-resistant obese adults relative to equally obese insulin-sensitive controls. *J. Clin. Endocrinol. Metab*. 89, 1630-5.

[30] Weickert, M.O., Mohlig, M., Koebnick, C., Holst, J.J., Namsolleck, P., Ristow, M., Osterhoff, M., Rochlitz, H., Rudovich, N., Spranger, J., and Pfeiffer, A.F. (2005). Impact of cereal fibre on glucose-regulating factors. *Diabetologia* 48, 2343-53.

# Chapter 8: Conclusions

## 8.1 Discussion

The main findings of this study were: (1) rutin and quercetin are not the compounds responsible for inhibition of glucose uptake observed upon treatment with a concentrate prepared from common buckwheat, (2) a solvent extract prepared from common buckwheat contains the same bioactives as the BWC based on the fact similar processes are stimulated by both (i.e. phosphorylation of p42/44 ERK and inhibition of 2-DOG uptake), (3) inhibition of 2-DOG uptake by the BWE is reversible, likely competitive and unique to common buckwheat, (4) the BWE is capable of inhibiting 2-DOG uptake by Caco2 cells, which leads to reduced transepithelial glucose transport, (5) the glucose uptake inhibitory activity of the BWE is present in one fraction after separation by HPLC, (6) the BWE stimulates phosphorylation of CREB independent of PKA, p90RSK or MSK-1 activation, (7) Clone 9 liver cells express GLUT3, which translocates to the plasma membrane in response to insulin treatment, (8) consumption of a food product made from whole grain common buckwheat flour containing 50 grams of available carbohydrate does not affect post-prandial glucose or insulin concentrations in healthy individuals or those with T2DM, but does modify post-prandial concentrations of selected gastrointestinal satiety hormones, (9) significant relationships between post-prandial responses of certain gastrointestinal satiety hormones after buckwheat consumption exist, (10) significant correlations between certain gastrointestinal hormones are affected by the presence of T2DM.

The current study was designed with two main overall objectives: to determine the pathways and mechanisms contributing to reduced glycemia that had been observed after treatment with a BWC [1], and to determine if acute regulation of post-prandial

glucose concentrations contributes to the improvements in glycemia that have been observed with long-term buckwheat consumption. The increasing incidence and prevalence of T2DM, despite a parallel increase in the number of pharmaceutical options available to treat this disease [2], highlights the need for complementary therapies for glycemic management. Discovering the molecular mechanisms involved in the regulation of glucose metabolism by buckwheat may facilitate the development of functional food products enriched in anti-hyperglycemic compounds, or nutraceuticals to aid in glycemic management. Research of this type would benefit not only those with T2DM, but also buckwheat farmers and the agriculture sector by adding novel value to a traditional food crop.

Inositol phosphoglycans containing D-CI have been shown to lower serum glucose levels in rats [3,4], presumably due to their insulin mimetic actions [5-7]. Since buckwheat contains relatively high amounts of D-CI [8], it was hypothesized that reduced serum glucose concentrations observed upon administration of a concentrate prepared from common buckwheat to rats resulted from the insulin mimetic properties of D-CI [1]. However, the current study has demonstrated that the BWC does not activate insulin signaling intermediates in H4IIE cells and therefore glucose-lowering properties are likely not due to the presence of this compound and its associated beneficial effects on insulin signaling (Chapter 4). Furthermore, the current study has also demonstrated that rutin, the major flavonoid in buckwheat [9], and its aglycone form quercetin, two compounds that reportedly inhibit glucose uptake [10], are not the compounds responsible for inhibiting glucose uptake upon treatment of H4IIE cells with the BWC. Activation of MAPK signaling, specifically increased phosphorylation of p38 MAPK and p42/44 ERK, was observed upon treatment of H4IIE cells with the BWC. However, use of chemical inhibitors demonstrated that activation of these pathways was not related to the glucose uptake inhibitory activity (Chapter 4). Furthermore, we also demonstrated

that rutin and quercetin are not the compounds responsible for activation of p42/44 ERK by buckwheat. Therefore, activation of other pathways by buckwheat must be responsible for inhibited glucose uptake.

Inhibition of glucose uptake by the BWC and BWE was an unexpected finding given the initial hypothesis of enhanced insulin signaling, and presumably consequent increased glucose uptake into peripheral tissues, as the mechanism for reduced blood glucose concentrations that had been observed previously. However, if inhibition of glucose uptake occurred in the small intestine, this could represent a potential mechanism contributing to the reduction in glycemia with buckwheat treatment. Therefore, we sought to determine if the glucose uptake inhibitory actions of buckwheat could be observed in Caco2 cells, a cell line often used to model absorptive mechanisms of the intestinal epithelium (Chapter 5). Indeed, buckwheat treatment elicited a significant reduction in glucose uptake and transepithelial glucose transport in this cell line. Although we were unable to identify the compound(s) responsible, the BWE glucose uptake inhibitory activity was present in only one fraction after HPLC (Chapter 5). Prior to the current study, limited information was available regarding potential mechanisms for improved glycemia observed with buckwheat consumption, and even less information on the effects of common buckwheat, the species of buckwheat used in the current study. We are the first to report inhibition of cellular glucose uptake by an extract of common buckwheat. However, others have reported inhibition of sucrase activity *in vitro* and reduced blood glucose concentrations after an oral sucrose test upon treatment with an extract produced from common buckwheat bran [11]. Similar to results from the current study, this effect could not be attributed to inhibitory actions of rutin [11]. Therefore, novel compounds in common buckwheat may reduce post-prandial glucose concentrations by targeting several areas of intestinal carbohydrate metabolism.

Activation of CREB, a transcription factor influencing transcription of gluconeogenic enzymes was stimulated by buckwheat, but not through the classical pathway involving activation of G-protein coupled receptors, increases in cAMP and phosphorylation by PKA (Chapter 5). Use of inhibitors of other known activators of CREB suggested MSK-1 as the candidate kinase responsible for increased phosphorylation of CREB, and although MSK-1 phosphorylation on certain amino acids was stimulated by buckwheat, gene silencing experiments did not support the role of MSK-1 in CREB phosphorylation elicited by the BWE. The functional significance of enhanced MSK-1 signaling by buckwheat is unknown, but is related to the phosphorylation of p42/44 ERK that we have observed in H4IIE cells, and therefore may involve mitogenic effects. A summary of the effects of the BWE on cellular glucose metabolism and signaling can be found in Figure 8.1.

Phytochemicals, particularly flavonoids, inhibit glucose uptake through direct inhibition of GLUT and SGLT transporters [10,12]. The observed inhibition of glucose transport by the BWE in the absence of changes in major signaling pathways of glucose metabolism (Chapter 4 and Appendix A Figure A-5 to A-8), prompted us to explore the possibility that compounds in buckwheat directly inhibit the activity of individual glucose transporters. Clone 9 cells were of particular interest to us because numerous publications report sole expression of GLUT1 in this cell line [13-15]. We proposed to develop a mammalian cell model in which to study the inhibitory effects of buckwheat on individual glucose transporters by co-transfecting Clone 9 cells with shRNA directed against GLUT1 and expression vectors containing genes for the other glucose transporters. While attempting to confirm by qPCR that only GLUT1 was expressed in Clone 9 cells, we discovered that these cells also express the GLUT3 gene (Chapter 6). Furthermore, GLUT3 protein is present in these cells, and responds to insulin stimulation with translocation to the plasma membrane. We are the first to report the presence of

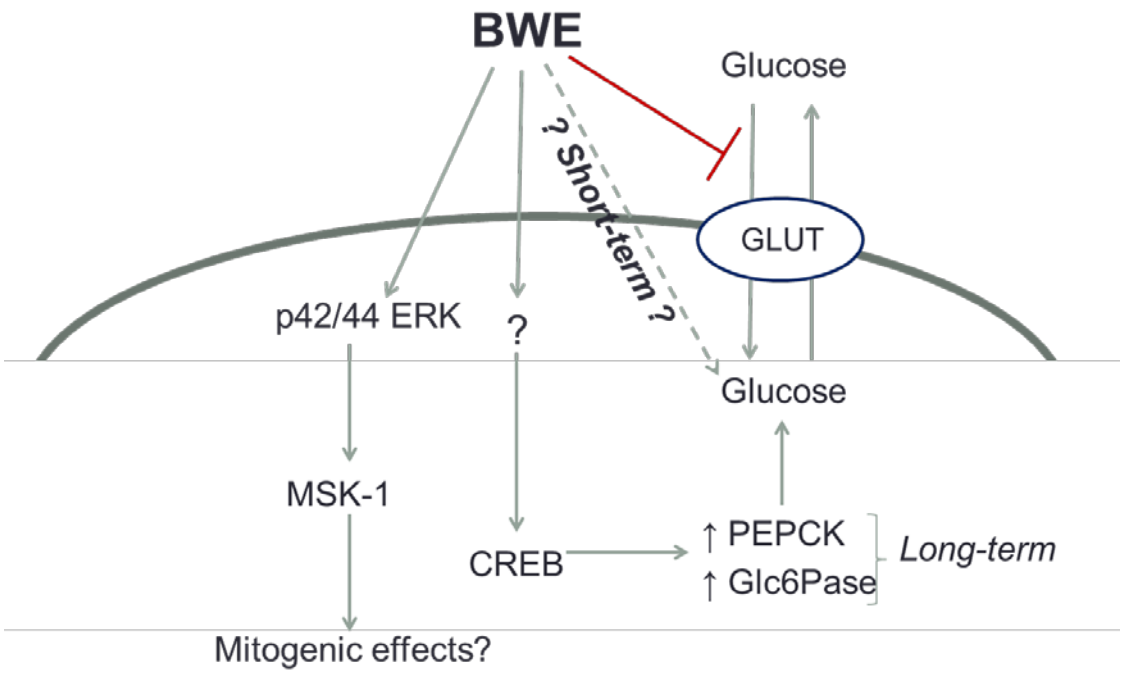


**Figure 8.1. A summary of the effects of the BWE on cellular glucose metabolism.**

insulin-responsive GLUT3 in Clone 9 cells, and although these cells could not be used as a model to study direct inhibition of individual glucose transporters by buckwheat, the discovery of GLUT 3 expression warrants a re-interpretation of results from previous studies claiming exclusive expression of GLUT1 in this cell line.

The ultimate goal of functional food research is to determine the efficacy and effectiveness of foods for disease prevention and/or management. Results from previous studies have demonstrated that incorporating baked products made from tartary buckwheat flour significantly reduces plasma glucose concentrations in individuals with T2DM [16,17]. However, based on our results in Chapter 5, tartary buckwheat does not possess the glucose uptake inhibitory activity that was observed in our common BWE extract. Furthermore, the limited studies conducted in humans do not establish whether reduced post-prandial glucose responses after consuming buckwheat contribute to long-term improvements in glycemia. To address this knowledge gap, we sought to determine the acute glucose-lowering effects of a cracker product made from whole grain common buckwheat flour on post-prandial glucose concentrations in both healthy individuals and individuals with T2DM (Chapter 7). While plasma glucose and insulin concentrations were not affected by consuming a cracker product made from whole grain common buckwheat flour in either group, a few points must be considered before concluding that buckwheat is ineffective at modifying acute glycemia. The lack of effect may have been due to a number of reasons, including insufficient dose of the compound responsible for inhibition of glucose uptake, or potential interactions between the active compound and other ingredients in the cracker product. Regardless, we did observe modest changes in the post-prandial response of certain gastrointestinal hormones involved in glucose metabolism and satiety, and some interesting associations between gastrointestinal hormones in healthy individuals and those with

T2DM. For example, overall GLP-1 concentrations were higher after consumption of the buckwheat crackers, but only in healthy individuals. Conversely, overall post-prandial GIP concentrations were lower in both groups after consumption of buckwheat crackers, while the overall response of PP was higher after consumption of buckwheat crackers. Interestingly, we observed several significant relationships between fasting levels of gastrointestinal hormones that appeared to be affected by the presence of T2DM: the significant relationship between fasting GIP and GLP-1 was observed only in individuals with T2DM, while the significant relationship between fasting insulin and PYY was observed only in healthy individuals. However, certain correlations were not affected by disease state, as the relationship between GLP-1 and PYY was present in both healthy individuals and those with T2DM. Further molecular studies are required to determine how gastrointestinal hormones regulate each other's secretion, and how this regulation may be affected by insulin resistance and/or T2DM.

## 8.2 Summary and Implications

In summary, the findings presented in the current study suggest that one or more compounds in common buckwheat inhibit glucose uptake, although the mechanism for inhibition remains to be completely elucidated. However, inhibition of facilitative mechanisms of transport without the involvement of several pathways implicated in glucose uptake, such as p42/44 ERK, p38 MAPK, PKC, mTOR, AMPK and PI3K suggest that the mechanism may involve direct inhibition of glucose transporters, as has been observed with other phytochemicals. Fractionation of the BWE using HPLC demonstrated that the glucose uptake inhibitory activity is present in only one fraction. Modulation of glucose metabolism by buckwheat is not limited to inhibition of glucose uptake, as evidenced by other compounds in buckwheat being associated with enhanced glucose production and higher levels of gluconeogenic proteins. Although CREB phosphorylation was stimulated upon treatment with the BWE, the upstream kinase responsible for this remains unknown. Despite the presence of compounds with the potential to inhibit intestinal glucose uptake, a food product made from whole grain common buckwheat flour was ineffective at reducing post-prandial glucose and insulin concentrations in both healthy individuals and those with T2DM, although insufficient dose of the bioactive in the food product, or possible interactions with other ingredients in the product may account for the lack of effect. Further studies are required at the molecular level in order to identify the compound(s) responsible for inhibition of glucose uptake. Knowing the identity of the compound(s) will stimulate food development research to enrich a food product made from buckwheat with this compound, potentially enhancing the glucose-lowering effects of such a product for glycemic management.

Novel therapeutic approaches to treating chronic diseases such as T2DM are required, as side effects and non-compliance with current pharmacological therapies are challenges for effective glycemic management. This study investigated the use of

common buckwheat for regulation of post-prandial glycemia and explored potential mechanisms contributing to this effect. Results from this study have contributed to the current state of knowledge by demonstrating the presence of several bioactive compounds in common buckwheat with a range of effects on glucose metabolism. The larger contribution of this study to the field of nutrition is the discovery that individual compounds present in buckwheat have the potential to regulate cellular processes and signaling pathways involved in glucose metabolism, contributing to the evidence that buckwheat is a functional food.

## 8.3 Strengths and Limitations

**Strengths:**

- Use of H4IIE cells – H4IIE express GLUT2 and other components of hepatic glucose metabolism and insulin signaling; thus it was an appropriate cell line in which to investigate hepatic glucose metabolism.
- Use of the Caco2 Transwell® system for transport experiments - polarized Caco2 cells grown on membrane supports are a well-known, widely used model in which to study nutrient transport, and allowed us to link reduced glucose uptake to reduced transepithelial glucose transport, a functional assessment of nutrient absorption.
- Use of lentiviral shRNA knockdown of MSK-1 to assess if MSK-1 is responsible for CREB phosphorylation by the BWE.
- Verification of glucose transporters present in H4IIE and Clone 9 cells by measuring both mRNA and protein levels.
- The current study is the first to examine the acute glucose-lowering effects of a food product made from whole-grain buckwheat flour in humans, and examined the effects in both healthy individuals and those with T2DM.
- Individuals with T2DM in the human study managed their disease with diet alone, thereby reducing confounding effects of oral anti-hyperglycemic agents on the outcome variables.
- Use of a cracker product in the human study – the cracker product was a relatively simple food product with minimal ingredients, thus reducing the potential effects of other food ingredients. Furthermore, the amount of cracker given represents a reasonable, easily-achievable serving of buckwheat.

- In the human study, both genders were equally represented in both the healthy volunteers and those with T2DM.

**Limitations**

- Rutin and quercetin undergo significant metabolism in the small intestine to form glucuronidated, methylated and sulfonidated metabolites; therefore, it is possible that one of these metabolites is responsible for inhibiting glucose uptake and therefore reducing the glycemia that has been previously observed in animal studies. The current study did not test the effects of rutin and quercetin metabolites on glucose uptake.
- Since H4IIE cells express only GLUT glucose transporter isoforms, we were able to conclude that the BWE inhibits blocks glucose uptake by inhibiting facilitative glucose transport mechanisms. However, Caco2 cells express SGLT glucose transporter isoforms in addition to GLUT isoforms; therefore it is unknown if the BWE also inhibits SGLT-mediated glucose transport.
- Although we observed inhibited glucose uptake in intestinal cells, it is unknown if this effect would be observed in an intact intestinal system in the presence of metabolizing enzymes that may alter the structure and therefore activity of the unknown bioactives.
- The observed inhibition of facilitative glucose transport does not reveal the nature of inhibition (i.e. if bioactive compounds in buckwheat directly inhibit glucose transporters).
- Dose of buckwheat in the cracker product – although the amount of cracker product given to participants was based on a standard of 50 grams of available

carbohydrate, it is unknown if this dose contains enough of the unknown bioactive to inhibit intestinal glucose uptake.

- Although levels of selected satiety hormones were higher after consumption of buckwheat crackers, we did not conduct subjective assessments of satiety and therefore cannot say that these increases would have led to reduced food intake.

## 8.4 Future Directions

- Glucose uptake experiments conducted in *Xenopus laevis* oocytes expressing single glucose transporters to determine if the BWE directly inhibits glucose transporters.
- Transport experiments in Caco2 cells using α-methylglucopyranoside, a glucose analogue that is transported exclusively by SGLT glucose transporter isoforms.
- Further experiments to determine the identity and structure of the compounds responsible for the biological activities observed, including mass spectrometry and/or nuclear magnetic resonance experiments.
- Once the identity of the bioactive compounds are known, experiments to characterized the nature of inhibition (i.e. competitive versus non-competitive) should be conducted.
- Testing the effects of the BWE on cellular glucose uptake in renal proximal tubule cells, as chemical inhibitors of SGLT-2 in the kidney are currently being investigated as a pharmacological treatment of T2DM.
- Development of milling procedures or other strategies to enhance the concentration of the bioactive in flour.

- Dose-response studies in humans – creating food products with increasing amounts of buckwheat to determine if higher amounts of buckwheat could reduce post-prandial glycemia.
- Gene silencing experiments in Clone 9 cells – transfection of siRNA or viral infection of shRNA targeting GLUT3 in Clone 9 cells to determine the contribution of GLUT3 to glucose uptake in Clone 9 cells.

## 8.5 Literature Cited

[1] Kawa, J.M., Taylor, C.G., and Przybylski, R. (2003). Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. *J. Agric. Food Chem.* 51, 7287-91.

[2] El-Kaissi, S. and Sherbeeni, S. (2011). Pharmacological management of type 2 diabetes mellitus: an update. *Curr. Diabetes Rev.* 7, 392-405.

[3] Fonteles, M.C., Almeida, M.Q., and Larner, J. (2000). Antihyperglycemic effects of 3-O-methyl-D-chiro-inositol and D-chiro-inositol associated with manganese in streptozotocin diabetic rats. *Horm. Metab. Res.* 32, 129-32.

[4] Ortmeyer, H.K., Bodkin, N.L., Lilley, K., Larner, J., and Hansen, B.C. (1993). Chiroinositol deficiency and insulin resistance. I. Urinary excretion rate of chiroinositol is directly associated with insulin resistance in spontaneously diabetic rhesus monkeys. *Endocrinology* 132, 640-5.

[5] Field, M.C. (1997). Is there evidence for phospho-oligosaccharides as insulin mediators? *Glycobiology* 7, 161-8.

[6] Jones, D.R. and Varela-Nieto, I. (1998). The role of glycosyl-phosphatidylinositol in signal transduction. *Int. J. Biochem. Cell Biol.* 30, 313-26.

[7] Jones, D.R. and Varela-Nieto, I. (1999). Diabetes and the role of inositol-containing lipids in insulin signaling. *Mol. Med.* 5, 505-14.

[8] Horbowicz, M. and Obendorf, R.L. (1994). Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. *Seed Sci Res* 4, 385-405.

[9] Kreft, S., Knapp, M., and Kreft, I. (1999). Extraction of rutin from buckwheat (Fagopyrum esculent Munch) seeds and determination by capillary electrophoresis. *J. Agric. Food Chem.* 47, 4649-52.

[10] Johnston, K., Sharp, P., Clifford, M., and Morgan, L. (2005). Dietary polyphones decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Let.* 579, 1653-7.

[11] Osaka, T., Nil, Y., Tom take, H., Ito, T., Tasmania, A., Yamasaki, Y., Sausage, S., Edazawa, K., Tsutsumi, R., Shuto, E., Okahisa, N., Iwata, S., and Sakai, T. (2011). Extracts of common buckwheat bran prevent sucrose digestion. *J. Nutr. Sci. Vitaminol. (Tokyo)* 57, 441-5.

[12] Kwon, O., Eck, P., Chen, S., Corpe, C.P., Lee, J.H., Kruhlak, M., and Levine, M. (2007). Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J.* 21, 366-77.

[13] Wei, Z., Peterson, J.M., and Wong, G.W. (2011). Metabolic regulation by C1q/TNF-related protein-13 (CTRP13): activation OF AMP-activated protein kinase and suppression of fatty acid-induced JNK signaling. *J. Biol. Chem.* 286, 15652-65.

[14] Behrooz, A. and Ismail-Beigi, F. (1998). Induction of GLUT1 mRNA in response to azide and inhibition of protein synthesis. *Mol. Cell. Biochem.* 187, 33-40.

[15] Shetty, M., Loeb, J.N., Vikstrom, K., and Ismail-Beigi, F. (1993). Rapid activation of GLUT-1 glucose transporter following inhibition of oxidative phosphorylation in clone 9 cells. *J. Biol. Chem.* 268, 17225-32.

[16] Lu, C., Zu, J., Zho, P., Ma, H., Tong, H., Jin, Y., and Li, S. (1992). Clinical application and therapeutic effect of composite tartary buckwheat flour on hyperglycemia

and hyperlipidemia. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, pp 458-64.

[17] Wang, J., Liu, Z., Fu, X., and Run, M. (1992). A clinical observation on the hypoglycemic effect of Xinjang buckwheat. In: Lin R, Zhou M, Tao Y, Li J, Zhang Z (eds) *Proceedings of the 5th international symposium on buckwheat*. Agriculture Publishing House, Bejing, 465-7.

# Appendix A: Extra Figures

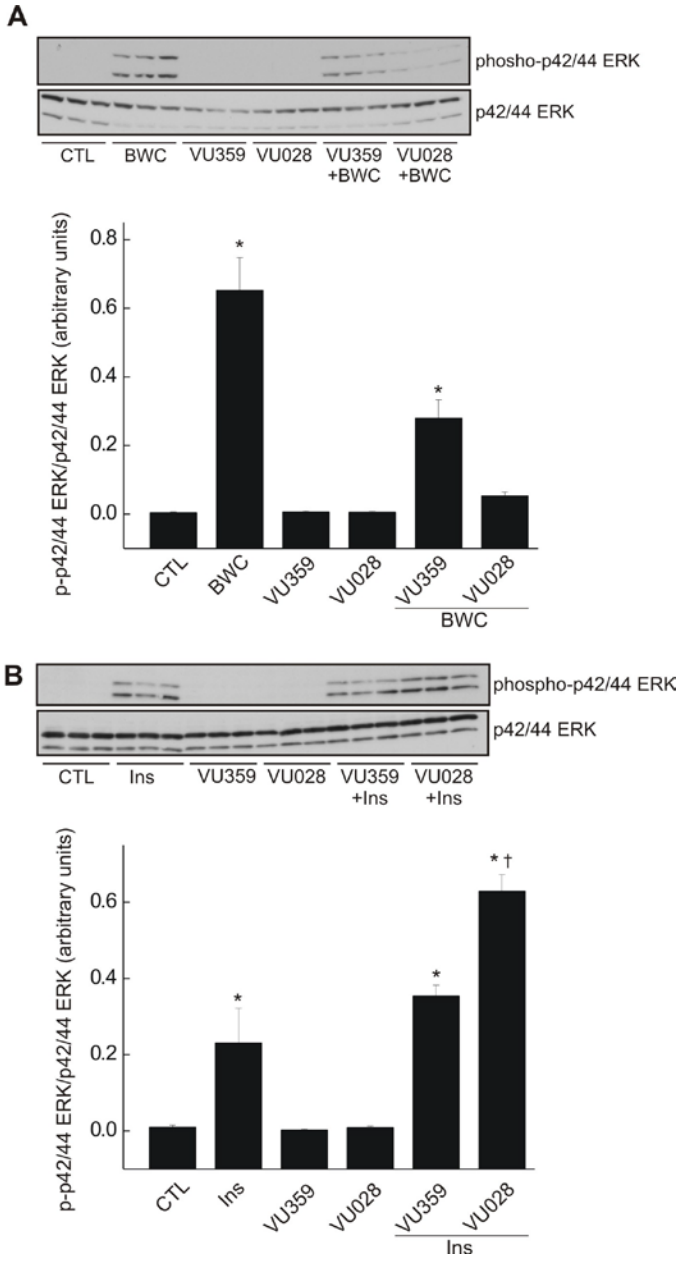


**Figure A-1. Phosphorylation of p42/44 ERK by buckwheat is dependent on phospholipase D (PLD) 2.** H4IIE cells were treated with 150 nM VU359595 (inhibits PLD1) or 3.75 µM VU0285655-1 (inhibits PLD2) for 10 minutes before addition of 0.25% v/v buckwheat concentrate (BWC; panel A) or 250 nM insulin (panel B) for 10 minutes. Relative intensities of the bands were quantified by densitometry and normalized to p42/44 ERK. * denotes significantly different from untreated control cells (CTL; $p<0.05$); † denotes significantly different from insulin (Ins; $p<0.05$). Data are presented as mean ± SEM (n=3).

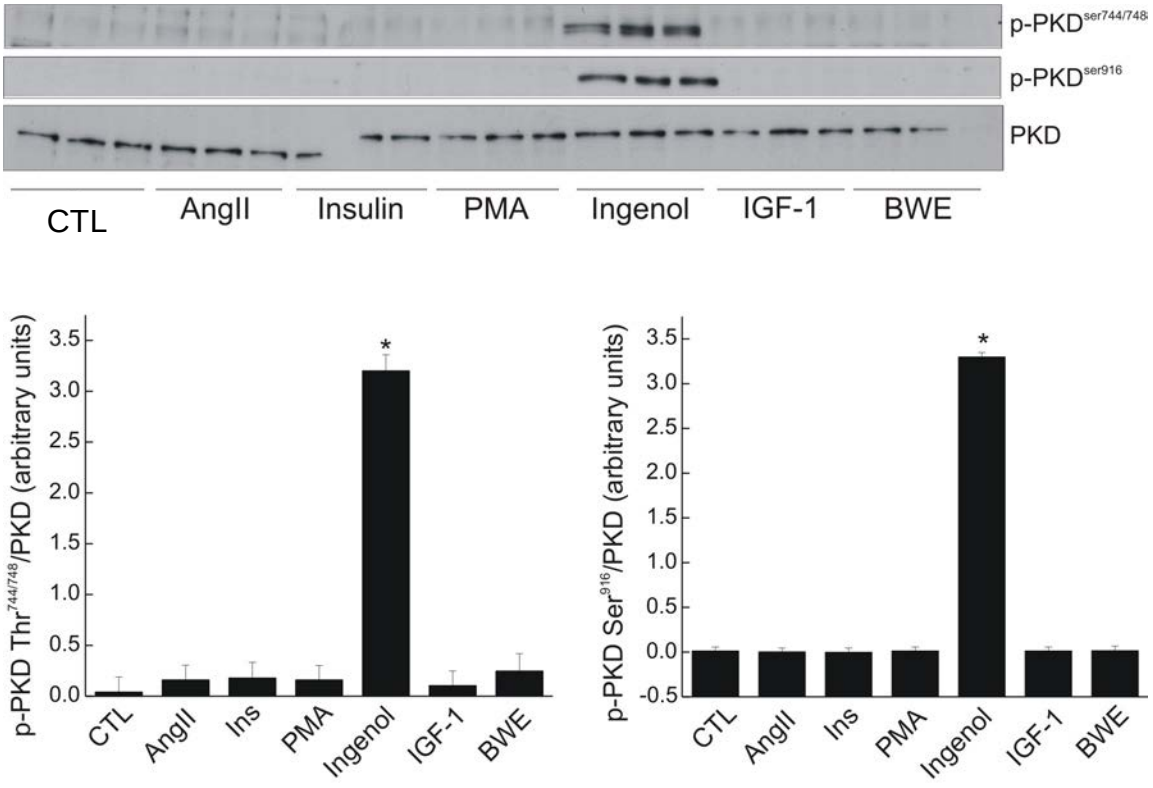


**Figure A-2. Phosphorylation of protein kinase D (PKD) on serine$^{744/748}$ and serine$^{916}$ is stimulated by Ingenol, but not the buckwheat extract.** H4IIE cells were treated with $10^{-5}$ M angiotensin II (AngII), 250 nM insulin (Ins), $10^{-9}$ M phorbol-12-myristate-13-acetate (PMA), $10^{-6}$ M ingenol, $10^{-6}$ M insulin-like growth factor-1 (IGF-1) or 0.25% v/v buckwheat extract (BWE) for 10 minutes. Phosphorylation of PKD Thr$^{744/748}$ and Ser$^{916}$ as well as non-phosphorylated PKD were assessed using Western blotting. Relative intensities of the bands were quantified by densitometry and normalized to PKD. *denotes significantly different from untreated control cells (CTL) as determined using one-way ANOVA with pre-planned contrast statements ($p<0.05$). Data are presented as mean ± SEM (n=3).

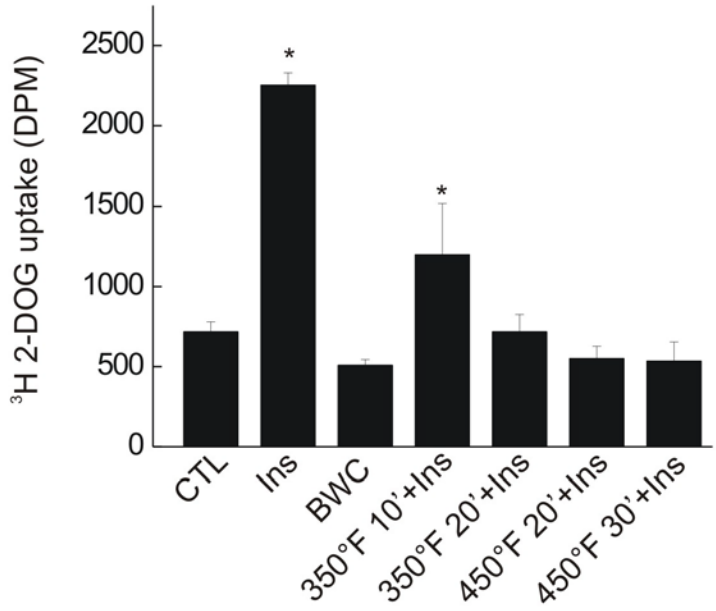


**Figure A-3. Glucose uptake inhibitory activity of buckwheat is heat stable.** Extracts were prepared from dehulled Koto buckwheat groats that were treated at the specified temperatures and times. H4IIE cells were treated with 0.25% v/v of these extracts in combination with 250 nM insulin for 10 minutes, before addition of 0.1 µCi/mL $^3$H 2-DOG. Insulin (250 nM) and buckwheat concentrate (BWC; 0.25% v/v) were used as controls. Inhibition of glucose uptake increased as treatment temperature and treatment time increased. Data are presented as mean ± SEM (n=3). * denotes significantly different from CTL ($p<0.01$) as determined using one-way ANOVA and pre-planned contrast statements. CTL= untreated cells; Ins = cells treated with 250 nM insulin; BWC = cells treated with 0.25% v/v buckwheat concentrate.

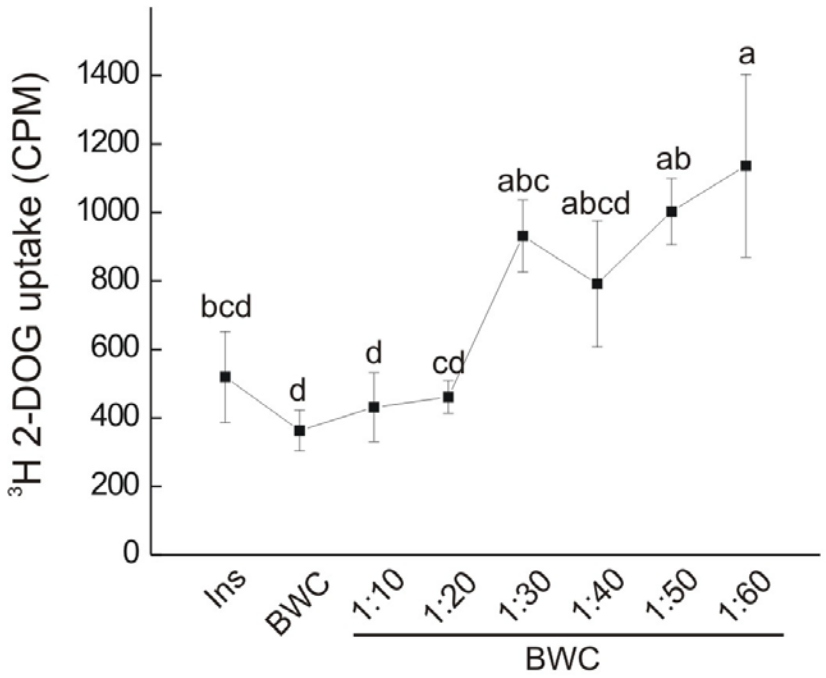


**Figure A-4. Inhibition of $^3$H 2-deoxy-D-glucose (2-DOG) uptake by the buckwheat concentrate is reduced by dilution.** H4IIE cells were treated with the indicated dilutions of buckwheat concentrate (BWC; prepared in water) in combination with 250 nM insulin (Ins) for 10 minutes before addition of 0.1 µCi/mL $^3$H 2-DOG. Inhibition of glucose uptake was still observed when the BWC was diluted 1:20, but was no longer present when the BWC was diluted 1:30. Data are presented as mean ± SEM. Means with different letters are significantly different as assessed using one-way ANOVA and Duncan's post-hoc test.

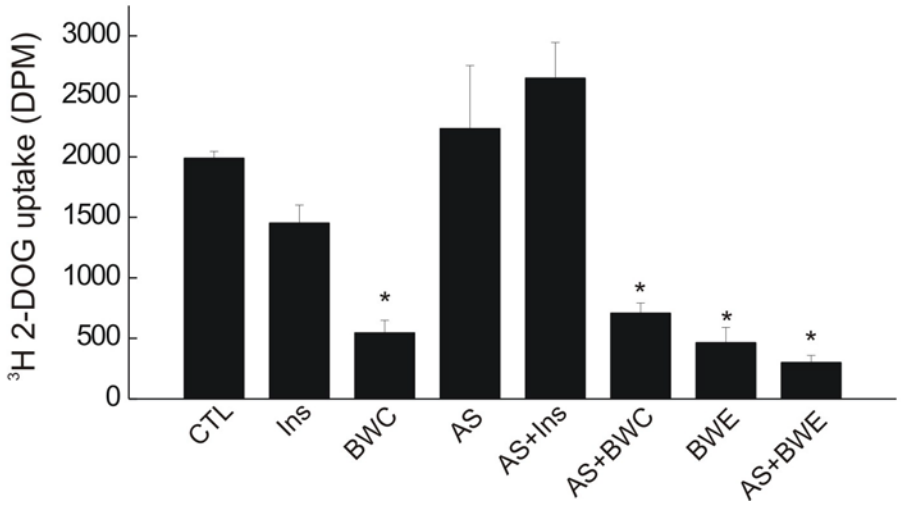


**Figure A-5. Inhibiting the PI3Kγ p110 subunit does not affect inhibition of $^3$H 2-deoxy-D-glucose uptake by buckwheat in H4IIE cells.** Cells were treated with $10^{-6}$ M AS605240 (AS) for 10 minutes before addition of 250 nM insulin (Ins) or 0.25% v/v of buckwheat concentrate (BWC) or buckwheat extract (BWE). Data are presented as mean ± SEM (n=3). * denotes significantly different from untreated CTL ($p<0.05$) as determined using one-way ANOVA and pre-planned contrast statements.

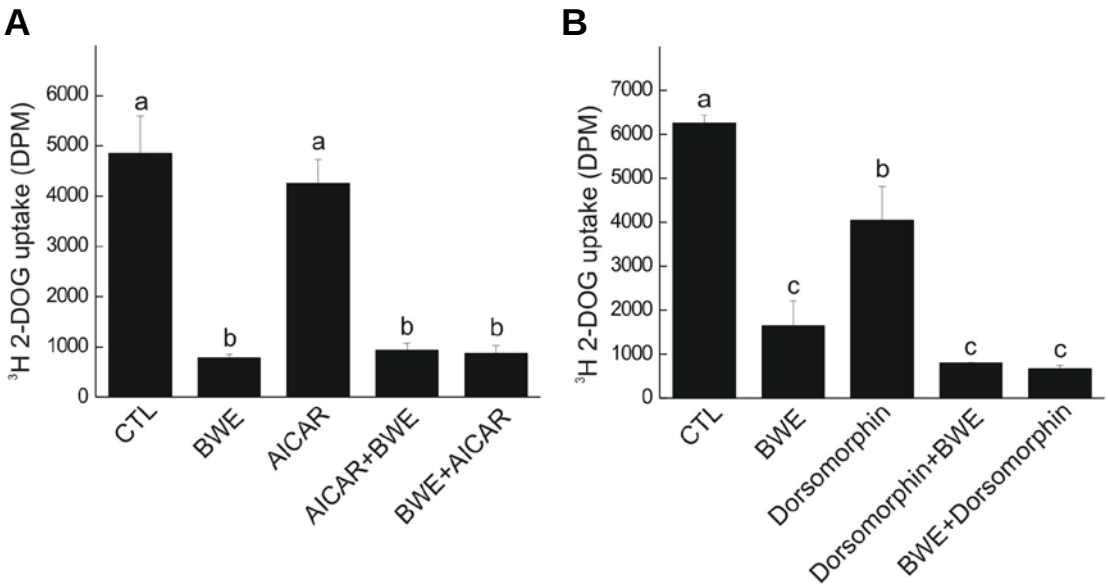


**Figure A-6. Inhibiting or activating AMP-activated protein kinase (AMPK) does not disrupt inhibitory actions of buckwheat on $^3$H 2-deoxy-D-glucose uptake.** H4IIE cells were treated with (A) $10^{-6}$ M AICAR (activates AMPK) or (B) dorsomorphin (inhibits AMPK) for 10 minutes before addition 0.25% v/v of buckwheat extract (BWE) for 10 minutes. Another set of cells was treated with BWE first, with addition of AICAR or dorsomorphin second. Data are presented as mean ± SEM (n=4). Means with different letters are significantly different as determined using one-way ANOVA and Duncan's post-hoc testing ($p<0.05$).

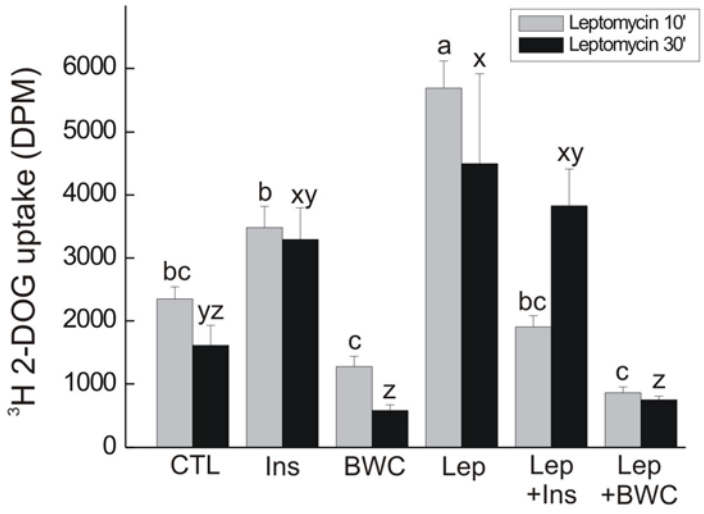


**Figure A-7. Inhibiting nuclear export with the use of leptomycin B does not affect inhibition of $^3$H 2-deoxy-D-glucose uptake elicited by buckwheat.** H4IIE cells were treated with 2 ng/mL leptomycin B (Lep) for 10 minutes (gray bars) or 30 minutes (black bars) before addition 0.25% v/v of buckwheat concentrate (BWC) for 10 minutes. Data are presented as mean ± SEM (n=4). Means with different letters are significantly different as determined using one-way ANOVA and Duncan's post-hoc testing ($p<0.05$); a, b and c are used for comparisons of 10 minute leptomycin B treatment groups; x, y and z are used for comparisons of 30 minute leptomycin B treatment groups.

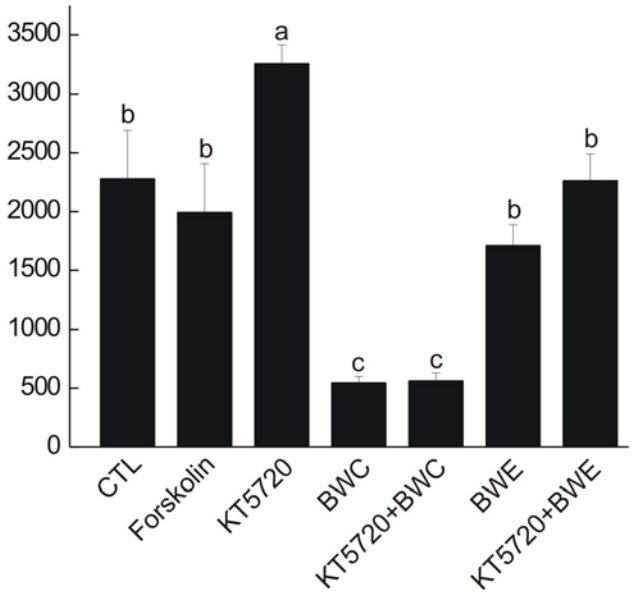


**Figure A-8. The effects of activation and inhibition of protein kinase A (PKA) on inhibition of $^{3}$H 2-deoxy-D-glucose uptake elicited by buckwheat.** H4IIE cells were treated with $10^{-2}$ M forskolin (PKA activator) or $10^{-2}$ M KT5720 (PKA inhibitor) for 10 minutes before addition of 0.25% v/v buckwheat concentrate (BWC) or buckwheat extract (BWE) for 10 minutes. Data are presented as mean ± SEM (n=3). Means with different letters are significantly different as determined using one-way ANOVA and Duncan's post-hoc means testing ($p<0.05$).

# Appendix B: The effect of Polybrene on viability of H4IIE cells

## Introduction

Polybrene (hexadimethrine bromide) is a cationic polymer used to enhance the efficiency of viral infection in cell culture. By neutralizing the charge conferred by sialic acid residues present on the cell surface, polybrene reduces the charge repulsion of viral particles and allows them better access to cell surface receptors [1]. Therefore, polybrene is useful in viral transduction experiments, where high rates of viral transduction are required.

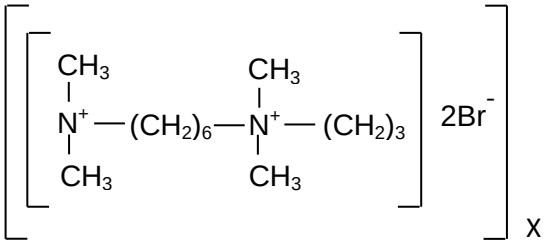

**Figure B-1.** Chemical structure of polybrene (hexadimethrine bromide)

Although Polybrene is commonly used at a working concentration of 1 to 10 µg/mL with little or no effect on cell viability, no information was available regarding the optimal dose of polybrene to use for viral transduction of H4IIE cells. Therefore, the objective of this experiment was to determine the concentration response effect of polybrene on cell viability in H4IIE cells.

## Methods

H4IIE hepatoma cells (ATCC catalogue #CRL-1548) were seeded into the wells of a 96-well tissue culture-treated plate (Nunc) at the following densities: 5000 cells/well,

10 000 cells/well and 20 000 cells/well. Cells were maintained at 5% $CO_2$ and 37°C for 24 hours, after which polybrene at concentrations ranging from 1 to 10 µg/mL was added to the wells. Cells were incubated with polybrene for 24 hours, after which cell viability was determined using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Burlington, ON, Canada).

## Results

When seeded at a density of 5000 cells/well, 1 µg/mL polybrene resulted in a dramatic reduction in the number of live cells. When seeded at a density of 10 000 cells/well, a reduction in the number of viable cells was observed when cells were treated with 4 µg/mL polybrene. Finally, when cells were seeded at 20 000 cells/well, polybrene treatment reduced the number of viable cells when used at a concentration of 5 µg/mL.

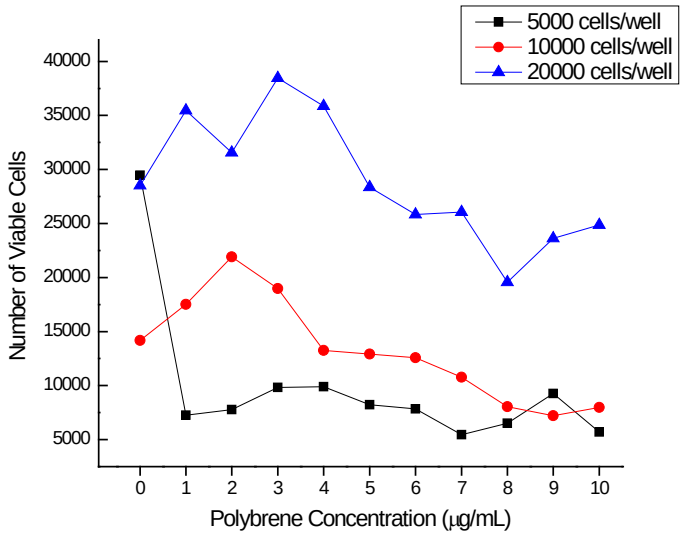


**Figure B-2.** Number of viable H4IIE cells after 24 hours of polybrene treatment

## Conclusions

The effects of Polybrene on the number of viable cells is dependent on cell density, with lower seeding densities more susceptible to toxic effects. Polybrene should not be used in H4IIE cells when seeding at a density of 5000 cells/well. However, when seeding H4IIE cells at densities of 10 000 and 20 000 cells/well, Polybrene may be used at concentrations of 3 and 5 µg/mL, respectively.

## Literature cited

[1] Davis, H.E., Rosinski, M., Morgan, J.R., Yarmush, M.L. (2004) Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. *Biophys J* 86,1234-42.

*Methods described in this appendix were used for experiments conducted in Chapter 5.*

# Appendix C. Detailed methods and optimization for lentiviral transduction of H4IIE cells

## Introduction

RNA interference is a post-transcriptional gene silencing pathway that uses small interfering RNA (siRNA) molecules to target specific mRNA transcripts for degradation, thereby suppressing gene expression and downstream protein production. Although cells contain endogenously produced siRNA, synthetic siRNA directed against a particular gene of interest can be made and introduced into cells. Once in the cell, the synthetic siRNA is processed and enters the RNA interference pathway in a similar manner as that of endogenously produced siRNA, thus allowing one to study the effects of selectively ablating that gene.

Delivery of synthetic siRNA into cells can be achieved using lipid-based transfection methods; however, certain cell types, such as H4IIE hepatomas, are resistant to traditional lipid-based transfection methods. In these cases, gene silencing can be achieved by infecting cells with lentiviruses genetically engineered to contain siRNA precursors, known as short hairpin RNA (shRNA). Since lentiviruses employ the infection mechanisms of retroviruses and use reverse transcriptases and integrases to insert their RNA-based genome into the DNA of host cells, infecting cells with a lentivirus containing shRNA results in integration of the shRNA into the DNA of the host cell. The shRNA is now produced from the host cell's DNA through inherent transcriptional processes and is processed to siRNA for entry into the RNAi interference pathway. By integrating the shRNA into the host cell's genome, lentiviral delivery of shRNA ensures that each daughter cell arising from host cell division contains the DNA corresponding to

the shRNA of the gene of interest, thereby ensuring stable expression of the shRNA and stable knock down. While other viral transduction systems also integrate into the host genome of dividing cells, lentiviruses are able to integrate genetic material into the genomes of non-dividing cells, which provides a significant advantage when examining quiescent cells [1]. Integrating the sequence for a reporter gene, such as green fluorescent protein (GFP), into the lentiviral genome allows one to assess the efficiency with which the cells are transduced, as successful entry of the virus into the host cell will result in transcription and production of GFP.

## Materials and Reagents

- H4IIE rat hepatoma cells (ATCC catalogue no. CRL-1548)
- α-modified Minimum Essential Media (Life Technologies, catalogue no. 11900-073)
- Nunc delta surface 96-well microplates (Fisher Scientific, catalogue no. 1256566)
- SMARTchoice lentiviral shRNA particles, set of 3 clones, with a Turbo GFP reporter (Thermo Scientific, catalogue no. SK-004665-00-10)
- Polybrene (Sigma-Aldrich, catalogue no. 107689)
- 1x PBS
- 2x sample buffer

## Procedure

Cells for transduction experiments were used between passage 5 and 10. Cells were seeded into the wells of a 96-well tissue culture-treated plate at a density of 20 000

cells per well in a total volume of 100 µL. The plate containing the cells was placed in a 37°C incubator with 5% $CO_2$ for 24 hours.

All viral work was conducted in a level 2 biosafety cabinet, with pipettes designated for viral work. Viral particles were removed from the -80°C freezer and thawed on ice. Each well of cells to be transduced required 20 µL of diluted viral particles plus 80 µL of α –MEM supplemented with Polybrene to achieve a final concentration of 3 µg/mL (i.e. 3 µg/mL Polybrene in a total volume of 100 µL). Sufficient viral particles were prepared to transduce each of the three clones in triplicate to assess level of silencing after 72 hours and 96 hours (i.e. 6 preparations per clone). Viral particles were diluted in pre-warmed α-MEM to achieve a multiplicity of infection of 50 transducing units/cell. Media was aspirated from the cells and 20 µL of the viral particles and 80 µL of the Polybrene-supplemented media was added to each well. The plate was placed in the incubator and incubated with the viral particles for 18 hours, after which the transduction media was aspirated and replaced with 100 µL of pre-warmed α-MEM.

Seventy-two and 96 hours after the initial transduction, cells were exposed to UV light at a wavelength of 488 nm to excite GFP. Pictures were taken in 3 separate fields of each of the triplicate wells for each clone, and the number of GFP positive cells was counted. Bright field images of each of these fields were also captured, and the total number of cells per field was counted. To calculate transduction efficiency, GFP-positive cells were expressed relative to the total number of cells. After pictures were taken, medium was aspirated and cells rinsed 3 times with 1x PBS. Cells were lysed in 70 µL 2x sample buffer and were assayed for protein using the BCA protein assay method. Degree of silencing was assessed by Western blotting using antibodies directed against MSK-1.

## Results

Representative images of GFP-expressing cells for each clone 72 and 96 hours post-transduction are presented in Figure B-1A. Overall, transduction with clone 1 resulted in a transduction efficiency of 70.4%, while transduction with clone 2 and clone 3 resulted in average transduction efficiencies of 70.3% and 69.7%, respectively. MSK-1 protein levels were reduced to the greatest extent compared to non-transduced cells by clone 3 96 hours after transduction (Figure B-1B).

## Conclusion

Transducing H4IIE cells with clone 3 at an MOI of 50 TU/mL produced an average efficiency of transduction of 70% and resulted in the lowest level of MSK-1 protein. Future MSK-1 silencing experiments should employ the transduction methods used in this optimization experiment to yield the highest degree of MSK-1 silencing in H4IIE cells.

## Literature Cited

[1] Manjunath N, Wu H, Subramanya S, Shankar P. (2009). Lentiviral delivery of short hairpin RNAs. *Adv Drug Deliv Rev* 61, 732-45.

*Methods described in this appendix were used for experiments conducted in Chapter 5.*

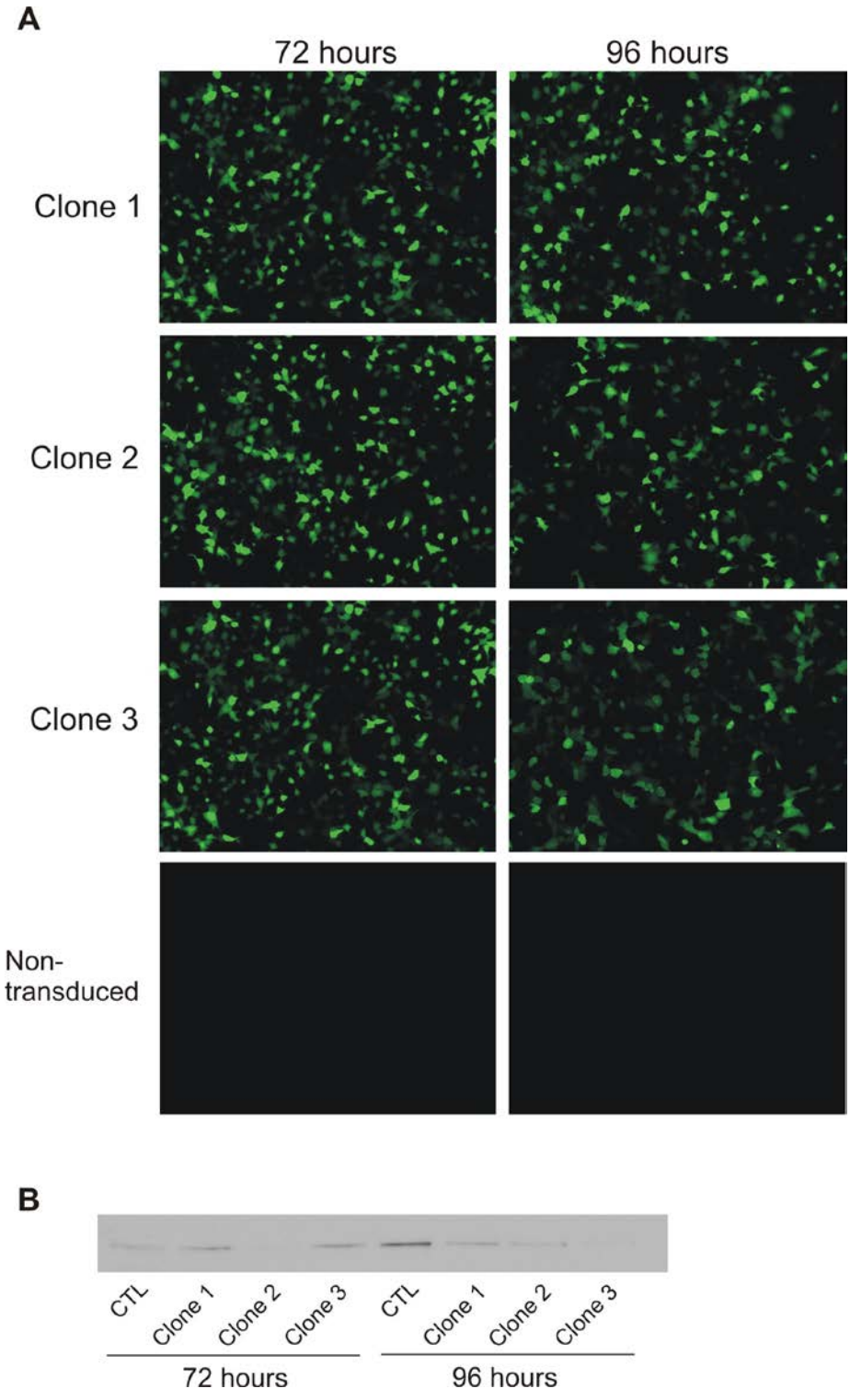


**Figure C-1.** (A) Representative images of GFP-positive H4IIE cells 72 and 96 hours after transduction with shRNA directed against MSK-1. (B) Protein levels of MSK-1 72 and 96 hours after lentiviral transduction with clone 1, clone 2 and clone 3.

# Appendix D: Detailed methods for RNA extraction, reverse transcription and optimization of quantitative PCR (qPCR)

## RNA Extraction

### Introduction

RNA was extracted from cell lines and tissues using E.Z.N.A. Total RNA kits (Omega Bio-Tek, Norcross, GA). These kits use the reversible binding properties of a silica-based spin technology known as HiBind® to reversibly bind RNA. Cell lysis is conducted under highly-denaturing buffer conditions to inactivate RNAses and ensure isolation of intact RNA. After adjustment of buffer conditions, cell debris is pelleted by centrifugation, and ethanol is added to the cleared lysate to provide appropriate binding conditions. The lysate/ethanol mix is loaded onto the E.Z.N.A. RNA column and then centrifuged, pulling the samples through the column. The RNA binds to the HiBind® column, and contaminants are washed away by applying wash buffers to the column and centrifuging. The final centrifugation step uses RNAase-free water to elute the RNA.

### Materials and reagents:

- E.Z.N.A. RNA extraction kits (Omega Bio-Tek, Norcross, GA)
    - Total RNA Kit I (catalogue # R6834-02)
    - Total RNA Kit II (catalogue # R6934-02)
    - Tissue RNA Kit (catalogue # R6688-02)
- Homogenizer spin columns and 2 mL collection tubes (Omega Bio-Tek, catalogue no. HCR003)
- RNase-free DNase set (Omega Bio-Tek, catalogue no. E-1091)
- Cell scrapers (Fisher Scientific, catalogue no. 08-100-241)

- β-mercaptoethanol (OmniPur® 2-Mercaptoethanol catalogue no. 6010, EMD Chemicals, Philadelphia, PA)
- Chloroform (Fisher Scientific, catalogue no. C607)
- 1 x PBS
    - 137 mM NaCl
    - 2 mM KCl
    - 10 mM $Na_2HPO_4$
    - 3 mM $KH_2PO_4$
- 70% ethanol

**Procedure**

For all RNA extractions, steps were performed at room temperature on benchtops designated exclusively for RNA extractions. Pipettes and microfuge tubes used were designated for RNA work only. Benchtops, equipment and supplies were thoroughly wiped with 70% ethanol before working with RNA. Cells to be used for RNA extractions were cultured on 100 mm tissue culture dishes.

*H4IIE, Clone9, Caco2, Day 0 and Day 2 3T3-L1 adipocytes.* Total RNA was extracted using the E.Z.N.A.$^{TM}$ Total RNA Kit I. When cells reached the desired confluence (80% for H4IIE, Clone 9, and Caco2 cells; 60% for Day 0 and Day 2 3T3-L1 adipocytes), media were aspirated from tissue culture dishes and cells rinsed 2 times with cold 1x PBS. Cells were then lysed in 700 µL TRK lysis buffer (provided in the kit) containing 2% v/v β-mercaptoethanol. The lysate was collected with a plastic cell scraper, transferred to a homogenizer mini column placed in a 2 mL collection tube and centrifuged for 2 minutes at 17 000 *g*. An equal volume of 70% ethanol was added to

the homogenate, which was mixed by gentle pipetting, applied to a HiBind® RNA column inserted into a 2 mL collection tube and centrifuged at 10 000 *g* for 60 seconds. The flow-through was discarded, 250 µL of RNA wash buffer I was added to the column, which was then centrifuged at 10 000 *g* for 1 minute. An on-column DNase digestion was performed to eliminate contaminating genomic DNA using the RNase-free DNase set. One and a half µL of RNase-free DNase (30 Kunitz) was added to 73.5 µL E.Z.N.A. DNase I Digestion Buffer, and this was added directly to the filter on the column containing the RNA. The column containing the DNase was incubated at room temperature for 15 minutes, after which 500 µL of RNA Wash Buffer I was added. The column was centrifuged at 10 000 *g* for 1 minute, followed by two sequential 1 minute centrifugations at 10 000 *g* with 500 µL RNA Wash Buffer II for to wash the column. The column was dried by centrifuging at 17 000 *g* for 2 minutes. RNA was eluted from the column by adding 40 µL of DEPC-treated water and centrifuging at 17 000 g for 2 minutes. To increase RNA yield and concentration, the elution centrifuging step was repeated using the first eluate. Eluted RNA was stored at -80°C.

*Day 4 and Day 8 3T3-L1 adipocytes.* RNA was isolated using the E.Z.N.A. Total RNA Kit II, which is designed specifically for isolating RNA from tissue or cells rich in fat, such as adipose tissue. Media were aspirated off of the cells, and 1 mL of RNA Solv reagent containing 2% v/v β-mercaptoethanol was added to each plate. Cells were scraped off the plate using a cell scraper, and centrifuged in a homogenizer mini column placed in a 2 mL collection tube for 2 minutes at 17 000 *g*. The homogenate was incubated at room temperature for 5 minutes, after which 200 µL of chloroform was added. The homogenate/chloroform mixture was vortexed for 60 seconds, incubated at room temperature for 3 minutes, and then centrifuged at 12 000 *g* for 15 minutes at 4°C to separate the aqueous phase (containing the RNA) and the organic phase. The upper

aqueous phase was transferred to a new 1.5 mL microfuge tube, and an equal volume of 70% ethanol was added. All subsequent steps were the same as RNA extraction using the E.Z.N.A. Total RNA Kit I.

*L6 myoblasts and myotubes.* RNA from L6 myoblasts and myotubes was isolated using the E.Z.N.A. Tissue RNA Kit. The addition of an incubation step with a protease enzyme to the standard RNA isolation protocol reduces the amount of intact contractile proteins and connective tissue present in skeletal muscle cells, facilitating the isolation of RNA from fibrous tissues and cells.

Media were aspirated from cells and 600 µL of TRK lysis buffer containing 2% v/v β-mercaptoethanol was added to each plate. Cells were scraped off of the plate using a cell scraper, and applied to a homogenizer mini-column placed in a 2 mL collection tube. The protease, consisting of 590 µL of RNase-free water and 10 µL of OB protease (provided in the kit), was added to the homogenate. The resulting mixture was agitated with gentle pipetting and incubated at 55°C for 10 minutes. The homogenate was then centrifuged at 14 000 g for 5 minutes at room temperature. The resulting supernatant was transferred to a new 1.5 mL microfuge tube, and an equal volume of absolute ethanol was added. The resulting mixture was mixed by vortexing, and then applied to a HiBind® RNA spin column. All subsequent steps were the same as for the E.Z.N.A. Total RNA Kit I.

## Quantification and Determination of RNA Quality

### Introduction

Quantity and quality of RNA was assessed using the Experion™ RNA StdSens Analysis Kit (Bio-Rad) for the Experion™ electrophoresis system. This system employs a microfluidics chip containing a series of plastic wells and gel-containing microchannels

to separate RNA based on size. After priming the chip with a mixture of gel and fluorescent stain, samples are loaded into the wells of the chip. An RNA ladder, consisting of RNA fragments ranging in size from 200 to 6000 nucleotides, is also loaded onto the chip. Electrodes on the electrophoresis station make contact with the wells of the chip and create a current that moves the samples through the wells and into the microchannels containing the gel. As the RNA moves through the gel it comes in contact with the RNA stain, which intercalates with the RNA molecule. The current continues to move the sample through a separating channel and eventually through the path of a laser, which excites the RNA stain to produce a fluorescent signal. This signal is expressed as an electropherogram, or a plot of the fluorescence values versus migration time. Area under the peaks corresponding to the RNA fluorescent signal is used to quantify the amount of RNA. To estimate RNA concentration of each sample, the total area under curve for the sample peaks is compared to that produced by the RNA standards of known concentrations. In addition, RNA quality is measured by comparing the degree of degradation of the samples to a set of degraded RNA standards. By comparing the degree of degradation between the samples and the standards, the Experion software calculates an RNA Quality Index (RQI), a number ranging from 1 to 10, with 1 being the most degraded RNA profile and 10 being the most intact, highest-integrity RNA profile. The Experion software also provides the ratio of the absorbance at 260 nm over 280 nm for each sample.

**Materials and reagents**

- Experion automated electrophoresis station, including the Experion priming station and the Experion vortex station (Bio-Rad Laboratories, catalogue no. 701-7001)

- Experion RNA Std Sens Analysis Kit (Bio-Rad Laboratories, catalogue no. 700-7103):
- Experion RNA Std Sens chips (Bio-Rad Laboratories, catalogue no. 700-7153)
- 70% ethanol

**Procedure**

All benchtops, equipment and supplies were thoroughly wiped with 70% ethanol before use. Components of the RNA Std Sens Analysis Kit were equilibrated to room temperature. The RNA gel was filtered by centrifuging 600 µL of the gel through a spin filter tube at 1500 *g* for 10 minutes. Gel stain was prepared by combining 65 µL of the filtered gel with 1 µL of RNA stain and gently vortexing. The tube containing the gel stain was wrapped in foil to protect it from light. RNA samples and the RNA ladder were removed from the -80°C freezer and allowed to thaw on ice. Two µL of RNA ladder and 2 µL of RNA samples were transferred to RNase-free microfuge tubes and then denatured by heating to 70°C for 2 minutes. The samples and ladder were then cooled on ice for 5 minutes, and then centrifuged briefly to bring the contents to the bottom.

The RNA Std Sens chip was then primed by pipetting 9 µL of the filtered gel-stain into the well labeled “GS”. The chip was placed in the priming station, the pressure setting on the priming station was set to “B” and the time setting set to 1, and the start button pressed. After priming, the chip was removed from the priming station and the microchannels inspected for the presence of bubbles. If bubbles were detected, the chip was discarded and a new chip was primed.

The primed chip was then placed on a clean surface for loading. Nine µL of gel stain was pipetted into the other well labelled “GS”, 9 µL of the filtered gel was pipetted into the well labelled “G”, and 5 µL of the loading buffer was pipetted into each of the

sample and ladder wells. One µL of denatured ladder was added to the "L" well, and 1 µL denatured sample was added to the appropriate sample wells. The chip was then vortexed for 1 minute in the vortex station, and then placed in the electrophoresis station for RNA analysis. Only RNA samples with an RQI between 7.5 and 10 were used for reverse transcription.

## Reverse Transcription

### Introduction

For the purposes of qPCR analysis, RNA must be converted to complementary DNA (cDNA). RNA was reverse transcribed to cDNA using qScript cDNA Supermix (Quanta Biosciences). This supermix is provided as a 5x concentrated solution containing all necessary components (except RNA template) for first strand synthesis of cDNA including buffer, nucleotides, magnesium, random primers, RNase inhibitors, qScript reverse transcriptase (an RNase H(+) derivative of MMLV reverse transcriptase) and stabilizers. The optimal temperature for activation of MMLV reverse transcriptase is 42°C; inactivation of this reverse transcriptase requires temperatures of 85°C.

### Materials and Reagents

- qScript cDNA Supermix (Quanta Biosciences, catalogue no. 95048)
- RNase/DNase-free water (Ambion, catalogue no. AM993L)
- RNA template
- 0.2 mL flat-cap PCR tubes (Fisher Scientific, catalogue no. 14230225)

One µg of RNA was combined with 4 µL 5x qScript cDNA Supermix, and the volume was brought up to 20 µL with RNase/DNase-free water. The tube was flicked to

gently mix the contents and centrifuged briefly to collect the contents at the bottom of the tube. Reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., St. Bruno, QC, Canada) using the following program: 25ºC for 5 minutes; 42°C for 30 minutes; 85°C for 5 minutes. Tubes containing the reaction products were held at 4°C until transferred to -80°C for storage.

## qPCR

### Introduction

The principles of PCR are based on the ability of DNA polymerase to synthesize a new DNA polymer complementary to a template DNA strand. Because DNA polymerase can only add nucleotides to the 3'-OH group of an existing nucleotide, short oligonucleotides are required to act as primers to which the polymerase can begin synthesizing the new DNA strand. By using primers complementary to a gene of interest, only this gene will be replicated. Using cDNA synthesized from reverse transcription of RNA as the template allows measurement of the relative amounts of specific RNA transcripts present in the original sample [1].

Since the amount of cDNA corresponding to the gene of interest is relatively small, it must be amplified to facilitate detection. cDNA is combined with a DNA polymerase, deoxynucleotide triphosphates, divalent magnesium, and a set of primers that are complementary to the 3’ and 5’ ends of the DNA to be amplified (known as the forward and reverse primers). These components are heated to high temperatures to denature the double-stranded DNA. The temperature is then lowered, allowing the forward and reverse primers to anneal to the template cDNA. Finally, the temperature is changed to a suitable temperature to allow the DNA polymerase to add nucleotides to the primer and extend the new DNA strand. This 3-step process is known as a cycle,

and is repeated forty times to produce billions of copies of the original cDNA; these copies are known as amplicons.

Quantitative PCR differs from standard PCR in that the amplified DNA is detected as the reaction progresses in real time, versus product detection at the end of standard PCR. Real-time detection of PCR products is achieved with the use of fluorescent dyes that intercalate with double-stranded DNA, or primers that are labeled with a fluorescent reporter that fluoresces only after hybridization to their complementary DNA target. As more and more amplicons are produced, the fluorescent signal increases. The cycle threshold, or Ct value, is defined as the number of PCR cycles required for the fluorescent signal to cross a background threshold value. Ct values are inversely proportional to the amount of target cDNA in the sample (i.e. the lower the Ct value the greater the amount of target nucleic cDNA in the sample).

An advantage of qPCR is the ability to determine specificity of primers for the intended product by conducting melt curve analysis of the PCR products. After completion of the PCR cycles, the temperature is slowly increased while simultaneously measuring fluorescence. As the double-stranded DNA denatures, the fluorescent signal decreases. Plotting the negative first derivative of the change in fluorescence against temperature generates a graph with distinct peaks corresponding to the melting temperature of each product. Theoretically, well-designed primers will only hybridize to the intended cDNA target and produce a single amplicon, which would dissociate at one specific temperature and display as a single peak on the melt curve graph. However, more than one peak may appear on a melt curve graph, indicating non-specific amplification. Due to their small size, primer-dimers usually melt at lower temperatures than the desired product, whereas nonspecific amplification can result in PCR products that melt at temperatures above or below that of the desired product. PCR products can be tested for the presence of the desired amplicon by performing agarose gel

electrophoresis to compare the size of the PCR amplicon to the anticipated size of the desired amplicon.

**Procedure**

*Primer design, ordering and reconstitution.* Primers were designed using Primer Blast software (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences of the genes of interest were referenced from NCBI's nucleotide database (http://www.ncbi.nlm.nih.gov.proxy1.lib.umanitoba.ca/nuccore) and entered in the PCR template field. All other fields were kept at the default values, with the following exceptions:

- Primer parameters:
    - PCR product size: 70 to 150 base pairs
- Exon/intron selection
    - Exon junction span: primer must span an exon-exon junction

Once designed, primers were ordered from Integrated DNA Technologies (http://www.idtdna.com/site). The scale selected was 25 nmole, with standard desalting. Primers were received lyophilized, and reconstituted with an appropriate volume of RNase/DNase-free water to achieve a concentration of 100 µM. To minimize free-thaw cycles of the stock vials, five 10 µL aliquots of each primer were made before storing all primers at -20°C.

*Primer optimization.* Annealing temperature. Although the melting temperature of primers may be calculated, optimal annealing temperatures must be determined empirically by performing PCR assays where all reaction conditions, except the temperature during the annealing step, are kept constant. Optimal annealing temperature for each set of primers used for qPCR was determined using the temperature gradient PCR function of the Mastercyler® ep *realplex*$^2$. In this function, the

heating block is set to incubate each column of the 96-well PCR plate at a different temperature during the annealing step. Annealing temperatures from 57°C to 72°C were tested.

*Standard curve analysis.* Specificity of primers for their intended target is imperative for accurate PCR results. Theoretically, a PCR assay conducted under ideal reaction conditions results in a doubling of product during each amplification cycle. Efficiency of a PCR reaction refers to the amount of original template that is amplified during the reaction. To determine the efficiency with which a specific set of primers can amplify cDNA, a PCR assay is conducted using serial dilutions of template cDNA. The Ct values for each dilution are plotted against the dilution factor, producing a standard curve. The equation of the linear regression line, along with the correlation coefficient (r) or the coefficient of determination ($R^2$), can then be used to assess optimization of qPCR.

If 10-fold serial dilutions are used, exact doubling of product after each cycle will result in a spacing of 3.32 cycles between the Ct values for each dilution, making the slope of the regression line -3.32. If the slope of the regression line of the standard curve is -3.32, the efficiency of the reaction is 100% (i.e. 100% of the target cDNA was amplified). Reaction efficiencies between 90% and 105% are generally accepted as optimized PCR reactions.

The r or $R^2$ value of a standard curve represents the linearity of the data, measuring the variability across assay replicates and whether the amplification efficiency is the same for different starting template amounts. Ideally, standard curves for optimized primers should have r values higher than 0.990 or $R^2$ values above 0.980. Standard curve assays are also used to determine the ideal amount of template to include in PCR assays.

**Materials and Reagents**

- 100 µM forward and reverse primers
- PerfeCTa SYBR Green FastMix (Quanta Biosciences, catalogue no. 95072)
- RNase/DNase-free water (Ambion, catalogue no. AM993L)
- Twin.tec real time 96-well PCR plates (Eppendorf, catalogue no. 951022015)
- Master clear real time PCR adhesive film (Eppendorf, catalogue no. 951022115)
- Mastercyler® ep *realplex*$^2$ (Eppendorf, Hauppauge, NY).
- cDNA template

For both temperature gradient and standard curve assays, primers and SYBR Green FastMix (SYBR) were removed from the -20°C freezer and cDNA template removed from the -80°C freezer and placed on ice. The SYBR tube was wrapped in foil to protect the contents from light.

Temperature gradient. Since all reaction components are kept the same at each temperature tested in gradient PCR, a master mix was prepared in a volume sufficient to perform PCR reactions at 12 temperatures, in triplicate. Each reaction contained 5 µL cDNA template, 100 nM forward primer, 100 nM reverse primer, 10 µL SYBR, and a sufficient volume of RNase/DNase free water to bring the total reaction volume to 20 µL. For 12 different temperatures tested in triplicate, 800 µL of the master mix was prepared. Four hundred µL of SYBR, 200 µL cDNA, appropriate volumes of forward and reverse primers to achieve a working concentration of 100 nM, and a sufficient volume of RNase/DNase-free water to bring the total volume to 800 µL was combined in a 1.5 mL microfuge tube. The tube was inverted several times and flicked to mix, and then briefly

centrifuged to remove any liquid that had become trapped in the lid or on the sides of the tube. The 96-well PCR plate was placed on ice, and 20 µL of the master mix was pipetted into the wells of columns 1 through 12 and rows A, B and C only of the plate. The plate was sealed with adhesive film and centrifuged briefly to bring the contents of each well to the bottom. The plate was then placed in the Mastercycler® ep *realplex*$^2$, which was programmed for the denaturation step: 95°C for 2 minutes; annealing step: gradient for 30 seconds; extension step: 68°C for 1 minute. In addition, a melt curve step was included at the end of the PCR program to assess temperature-dependent specificities of the primers for their intended target. The ideal annealing conditions were selected by examining the Ct values for each of the temperatures tested. Similar Ct values amongst the tested temperatures indicate that the ideal annealing temperature is among those tested temperatures. The highest temperature amongst the test temperatures displaying similar Ct values was chosen as the ideal annealing temperature for subsequent PCR assays using these primers.

Standard curve. Six 10-fold serial dilutions of cDNA were prepared in RNase/DNase-free water. These dilutions were used as the template for PCR reactions, which were set up with the following components: 5 µL cDNA template, 100 nM forward primer, 100 nM reverse primer, 10 µL SYBR, and a sufficient volume of RNase/DNase free water to bring the total reaction volume to 20 µL. Each dilution was tested in triplicate, so a master mix with sufficient volumes for 3.5 reactions was prepared in 0.5 mL RNase/DNase-free microfuge tubes. The tube was inverted several times and flicked to mix, and then briefly centrifuged to remove any liquid that had become trapped in the lid or on the sides of the tube. The 96-well PCR plate was placed on ice, and 20 µL of the reaction was pipetted into the wells of the plate. The plate was sealed with adhesive film and centrifuged briefly to bring the contents of each well to the bottom. The plate was then placed in the Mastercycler® ep *realplex*$^2$, which was programmed as follows:

denaturation step - 95°C for 2 minutes; annealing – the primer-specific annealing temperature as determined from gradient PCR analysis; extension – 68°C for 1 minute. Assays with efficiencies between 90% and 105% with an $R^2$ value above 0.980 were considered optimized. Ideal template amount for subsequent PCR reactions was chosen by picking a template amount that yielded Ct values between 20 and 30.

**Literature Cited**

[1] Orlando, C., Pinzani, P., Pazzagli, M. (1998). Developments in quantitative PCR. *Clin Chem Lab Med*. 36, 255-69.

*Methods described in this appendix were used for experiments conducted in Chapter 4.*

# Appendix E: Detailed methods for subcellular fractionation using differential velocity centrifugation

## Introduction

Cellular organelles and compartments differ in both size and density and therefore can be separated based on the basis of sedimentation rate [1]. After disrupting cells in an isotonic buffer, cell homogenates are centrifuged at a relatively low speed to pellet cell debris, nuclei, mitochondria and plasma membranes, which can be further separated using sucrose cushions and higher speed centrifugation. The supernatant from the original centrifugation can be further centrifuged at higher speeds to separate high density and low density microsomal fractions. The resultant fractions can be prepared for SDS-PAGE and Western immunoblotting to assess protein tranlsocation between subcellular fractions.

## Materials and reagents:

- HES buffer:

  255 mM sucrose

  20 mM Hepes pH 7.4

  1 mM EDTA

- HE buffer:

  20 mM Hepes pH 7.4

  1 mM EDTA

- 1.12 M sucrose in HE buffer:

  38.34 g D-sucrose dissolved in 100 mL HE buffer

- Halt Protease inhibitor (100x) (Pierce, catalogue no. 87786)
- 2x sample buffer

  0.2 M Tris-HCl pH 6.8

  2% sodium dodecylsulfate

  30% glycerol
- Cell scrapers (Fisher Scientific, catalogue no. 08-100-241)
- Dounce homogenizer
- 6.5 mL (16 x 64 mm) thickwall polycarbonate ultracentrifuge tubes (Beckman Coulter, catalogue no. 355647)
- 13.2 mL (14 x 89 mm) ultraclear thinwall polyallomer ultracentrifuge tubes (Beckman Coulter, catalogue no. 344659)
- MLA-80 fixed angle rotor (Beckman Coulter)
- SW-41 Ti swinging bucket rotor (Beckman Coulter)

## Procedure

Cells were grown on 150 mm tissue culture plates, using three plates per treatment. All solutions were stored at 4°C to ensure they were cold at the time of use. Before use, 500 µL of Halt Protease Inhibitor Cocktail were added to 49.5 mL of HES buffer. Cells were placed on ice, media was aspirated and the cells rinsed 3 times with HES. Three mL of HES were added to one of the three plates and cells were scraped vigorously using a cell scraper. The cell suspension was transferred to the second plate, and again cells were scraped into the 3 mL of HES plus cells. This process was repeated for the third plate. The resulting combined cell suspension was homogenized with 20 strokes in a Dounce homogenizer, the homogenate was transferred to a thickwall polycarbonate ultracentrifuge tube and the homogenate was centrifuged at

19000 *g* for 20 minutes in an MLA-80 rotor (Beckman Coulter). The supernatant was transferred to a thickwall polycarbonate ultracentrifuge tube and centrifuged at 40 000 *g* for 20 minutes in an MLA-80 rotor (Beckman Coulter). The pellet from this centrifugation was the high-density microsomal fraction, and was resuspended in 100 µL of 2x sample buffer and stored at -20°C. The supernatant from this centrifugation was transferred to a thickwall polycarbonate ultracentrifuge tube and centrifuged at 180 000 *g* for 75 minutes in an MLA-80 rotor (Beckman Coulter). The pellet from this centrifugation was the low-density microsomal fraction, and was resuspended in 100 µL of 2x sample buffer and stored at -20°C.

The pellet from the initial 19 000 *g* centrifugation was resuspended in 1.5 mL of HES buffer, and was gently layered onto 6 mL of 1.12 M sucrose in a thinwall, ultra clear, polyallomer ultracentrifuge tube. The tubes were centrifuged at 100 000 *g* for 60 minutes in an SW-41 Ti swinging bucket rotor (Beckman Coulter), yielding a white interface (the plasma membrane) and a pellet (nucleus/mitochondria). This pellet was resuspended in 200 µL 2x sample buffer and stored at -20°C. The interface was carefully removed with a Pasteur pipette and transferred to a thickwall polycarbonate ultracentrifuge tube and centrifuged at 40 000 *g* for 20 minutes in an MLA-80 rotor (Beckman Coulter). The pellet from this final centrifugation was the plasma membrane, and was resuspended in 100 µL 2x sample buffer and stored at -20°C.

## Literature Cited

[1] Michelsen, U., von Hagen, J. (2009). Isolation of subcellular organelles and structures. *Methods Enzymol*. 463, 305-28.

*Methods described in this appendix were used for experiments conducted in Chapter 6.*

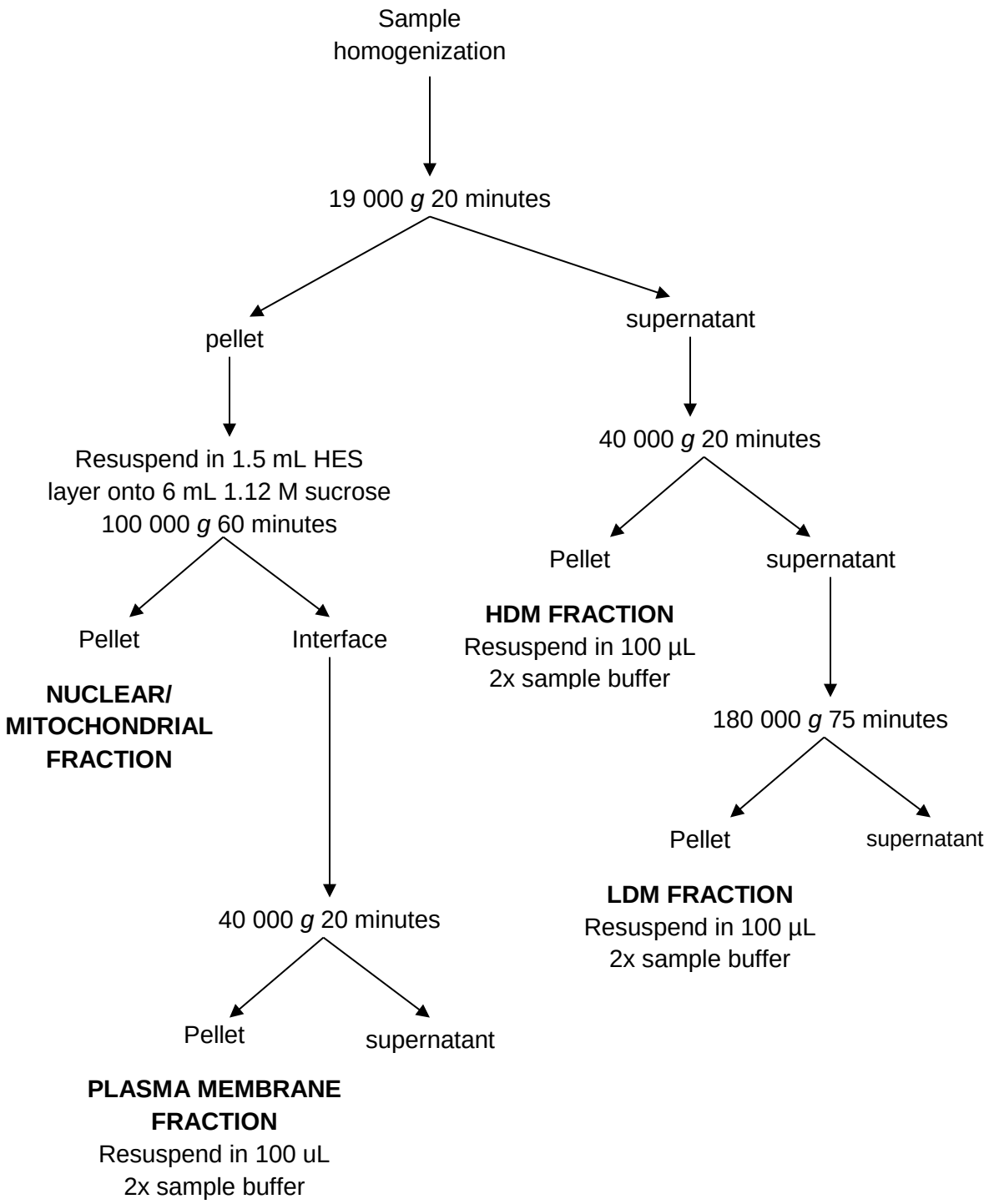


**Figure E-1. Overview of subcellular fractionation procedure.** HDM = high density microsomal fraction; LDL = low density microsomal fraction; PM = plasma membrane.

# Appendix F. Detailed methods for glucose transport experiments using Caco2 cells and Transwell® inserts

## Introduction

The intestinal epithelial layer is composed of a single layer of enterocytes that are responsible for absorption and transport of nutrients. If cultured under certain conditions, the human colon carcinoma cell line Caco2 will form monolayers with a differentiated phenotype mimicking many functions of the small intestinal epithelium, including transport of nutrients. As such, Caco2 cells grown on permeable filters have become a widely-accepted model with which to study intestinal nutrient absorption and transport [1].

To perform transport studies in Caco2 monolayers, cells must be grown to full differentiation and transporter expression on a membrane support. Transwell® inserts are composed of a sterile 0.4 µm polycarbonate filter which fit into the wells of a multi-well tissue culture plate. Placing the insert into the well forms an apical compartment and a basolateral compartment (Figure AE-1). Seeding cells in the upper compartment of the transwell support and growing to confluence results in the generation of a single layer of cells with fully-developed tight junctions, and thus only molecules that are absorbed and transported by the Caco2 cells will appear in the basolateral compartment. Integrity of the tight junctions in cell monolayers formed on transwell membranes can be determined by measuring transepithelial electrical resistance (TEER), or the resistance to pass current across a cell monolayer. TEER measurements can be made using a special probe attached to a voltohmmeter. Increasing confluence and tightness of monolayers results in higher TEER values. TEER values of transwells containing cells are referenced against a transwell containing no cells (a blank), and generally only

monolayers exceeding the blank TEER value by 400 Ω are considered of high enough integrity to use for transport experiements.

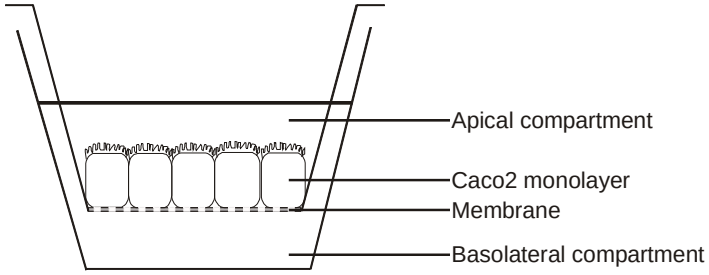


**Figure F-1.** Caco2 monolayer grown on a permeable filter support

## Materials and Reagents

- Caco2 colorectal carcinoma cells (ATCC catalogue no. HTB-37)
- 150 mm x 20 mm Nunclon™ Δ tissue culture dishes (Thermo Scientific, catalogue no. 168381)
- Seeding medium
    - 20% Fetal bovine serum (Gibco, catalogue no. 12483-020) in α-modified Minimum Essential Media (Life Technologies, catalogue no. 11900-073)
- Growth medium
    - 10% Fetal bovine serum (Gibco, catalogue no. 12483-020) in α-modified Minimum Essential Media (Life Technologies, catalogue no. 11900-073)
- 1x PBS (137 mM NaCl, 2 mM KCl, 10 mM $Na_2HPO_4$, 3 mM $KH_2PO_4$)
- 0.185% trypsin in HBS buffer
    - 8 mL 2.5% trypsin (Invitrogen, catalogue no. 19090-046)
    - 100 mL HBS buffer (20 mM Hepes pH 7.4, 150 mM NaCl)

- 24-well tissue culture plate with 6.5mm Transwell® inserts with 0.4µm pore polycarbonate membrane (Corning, catalogue no. 3413)
- Millicell electrical resistance system (Millipore, catalogue no. MERS 000 01)
- Hank's balanced salt solution (HBSS) pH 7.4 (137 mM NaCl, 5.4 mM KCl, 0.25 mM $Na_2HPO4$, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM NaHCO3)
- $^3H$ D-glucose (Perkin Elmer, catalogue no. NET331C005MC)
- 100 mM D-glucose

## Procedure

*Transwell® preparation and cell seeding.* Transwell inserts were prepared for seeding by adding 100 µL of seeding media (20% FBS in α-MEM) to the top of the transwell insert (apical compartment). The transwell insert was removed from the well using sterile forceps, and 600 µL seeding media was added to the wells of the tissue culture plate (basolateral compartment). The plate was then incubated at 37°C for 1 hour.

*Preparation of Caco2 cells for cell seeding.* Caco2 cells were grown to 80% confluence on 150 mm x 20 mm tissue culture plates in 20 mL seeding media. When cells were 80% confluent, media was aspirated and cells rinsed once with sterile PBS. Three mL of 0.185% trypsin in HBS buffer was added to the plate and cells were placed in the tissue culture incubator for cell detatchment. When cells were detatched, trypsin was inactivated by adding 3 mL seeding media to the plate, and cell clumps were disrupted with pipetting. Cells were then counted with a hemocytomer, collected in a sterile 15 mL conical tube, and centrifuged at 250 *g* for 10 minutes to pellet the cells. Cells were resuspended in seeding media to achieve a cell density of 112 000 cells/mL.

One hundred µL of the 112 000 cells/mL suspension was added to the apical compartment of the prepared transwell® plate. Transwell® plates were then placed at

37°C and 5% $CO_2$, and were cultured for 21 days to allow full differentiation and development of monolayers with tight junctions. On days 3, 6 and 9 post-seeding, media was aspirated from the basolateral compartment, 50 µL of growth media was added to the apical compartment and 600 µL fresh growth media was placed into the basolateral compartment. On days 12, 15, 18 and 20 post-seeding, media was aspirated from the basolateral compartment, followed by aspirating from the apical compartment, 100 µL fresh growth media was added to the apical compartment and then 600 µL fresh growth media added to the basolateral compartment. During all media changes, caution was taken to maintain net positive hydrostatic pressure above the cells by aspirating the media from the basolateral compartment first and the apical compartment second, followed by addition of media to the apical compartment first, followed by the basolateral compartment second. Monolayers were ready 21 days after initial seeding.

*TEER measurements.* Electrodes were sterilized by immersing the electrodes in 70% ethanol for 30 minutes, followed by air-drying for 15 seconds. The mode switch on the volthommeter was turned to R, and the power switch turned on. The electrodes were immersed so that the shorter electrode was in the apical side and the long probe into the basolateral compartment. TEER measurements were taken in a transwell® insert containing no cells. Transwell® inserts containing cells were used if their TEER values exceeded the blank insert by 400 Ω.

*$^3$H glucose transport assays*. Transwell® inserts were removed from wells with sterile forceps and held at an angle to decant medium. Inserts were then placed in the wells of a new 24-well plate containing 600 µL HBSS per well. One hundred µL of HBSS containing 0.25% or 0.5% v/v of the buckwheat extract was added carefully to the apical compartment by placing the tip of the pipette on the side of the insert and slowing dispensing the solution, and the plate was incubated at 37°C and 5% $CO_2$ for 10

minutes. The plate was then removed from the incubator, and 5 µL of 100 mM D-glucose and 2 uL of a 1/1000 dilution of 5 mCi $^{3}$H D-glucose were added. The final concentrations D-glucose and $^{3}$H D-glucose were 5 mM and 0.1 µCi, respectively. After incubation for 30 minutes at 37°C and 5% $CO_2$, the plate was removed from the incubator, and 100 µL of buffer was removed from the basolateral compartment and combined with 3 mL of scintillation fluid.

## Literature cited

[1] Stenberg, P., Norinder, U., Luthman, K., and Artursson, P. (2001). Experimental and computational screening models for the prediction of intestinal drug absorption. *J. Med. Chem.* 44, 1927-37.

*Methods described in this appendix were used for experiments conducted in Chapter 5.*