Characterization of urinary inositol excretion in diabetes mellitus and the antihyperglycemic effects of a buckwheat concentrate, containing D-*chiro*-inositol.

Julianne Marie Kawa

A thesis submitted to the Faculty of Graduate Studies
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Characterization of Urinary Inositol Excretion in Diabetes Mellitus and the Antihyperglycemic Effects of a Buckwheat Concentrate, Containing D-*chiro*-inositol

BY

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Abstract

Diabetes mellitus (DM) is a chronic disease and a leading cause of death in developed countries. DM is characterized by hyperglycemia, resulting from deficiencies in insulin secretion and levels and/or inadequate insulin action. Insulin mimetic compounds, containing D-*chiro*-inositol (D-CI) and *myo*-inositol (MI), have been identified as having altered bioactivity and metabolism in DM. The pattern of urinary excretion of D-CI and MI has also been reported as altered in DM, however, there are discrepancies regarding the direction of the change. In addition, administration of chemically synthesized free D-CI has demonstrated antihyperglycemic effects in DM, presumably in response to a correction of the altered activity and metabolism of these compounds. Buckwheat contains D-CI, MI, and derivatives of D-CI known as fagopyritols, and is a potential natural source of these compounds in treating DM. The present research investigated 1) the pattern of urinary D-CI and MI excretion in animal models and humans with DM, 2) the effects of a buckwheat concentrate (BWC) containing D-CI, MI, and fagopyritols on *in vivo* glycemic control, 3) the insulin-mimetic effects of the BWC on cell signal transduction proteins in H4IIE cells, and 4) the effects of a BWC on glucose uptake in H4IIE cells. The main findings of this research were that 1) urinary excretion of D-CI and MI was elevated in animal models and humans with DM, concomitant with hyperglycemia and glucosuria, 2) a BWC, containing D-CI, MI, and fagopyritols, had antihyperglycemic effects in an animal model of DM, 3) the BWC demonstrated insulin-mimetic effects by activating MAPK and $p70^{S6K}$ (Thr^{421}) in H4IIE cells, and 4) the BWC inhibited basal and insulin-stimulated

glucose uptake in H4IIE cells, whereas D-CI stimulated glucose uptake similar to insulin in these cells. Thus, elevated urinary D-CI and MI excretion is associated with hyperglycemia and glucosuria. This finding can contribute to the current understanding of DM and may be useful as a biomarker or lead to novel treatments for the disease. Although the components responsible are not known, the anti-diabetic properties of buckwheat suggest it may be useful in the management of DM which can have implications for the buckwheat industry.

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List of Abbreviations

AMPK	AMP-activated protein kinase
APS	ammonium persulfate
ATP	adenine triphosphate
BSA	bovine serum albumin
BWC	buckwheat concentrate
BW HIGH	buckwheat high dose
BW LOW	buckwheat low dose
CTRL	control
D-CI	D-*chiro*-inositol
DM	diabetes mellitus
DM-1	type 1 diabetes mellitus
DM-2	type 2 diabetes mellitus
FBS	fetal bovine serum
GIP	glucose-dependent insulinotropic polypeptide
GK	Goto-Kakizaki
GLUT	Na^+ independent glucose transporter
GLP-1	glucagon-like peptide-1
GPI	glycosylphosphatidylinositol
GSK-3	glycogen synthase kinase-3
$HbA1_c$	glycosylated hemoglobin
HRP	horseradish peroxidase
IGF/IR	insulin-like growth factor/insulin receptor

IGT	impaired glucose tolerance
INS	insulin
InsR	insulin receptor
IPG	inositol phosphoglycan
IPGTT	intraperitoneal glucose tolerance test
IRS	insulin receptor substrate
JAK	janus kinase
MAPK	mitogen activated protein kinase
MI	*myo*-inositol
mTOR	mammalian target of rappamycin
NADH	nicotinamide adenine dinucleotide
OGTT	oral glucose tolerance test
PDH	pyruvate dehydrogenase
PEPCK	phosphoenolpyruvate carboxykinase
PKA	cAMP-dependent protein kinase A
PLD	phospholipase D
PL HIGH	placebo high dose
PL LOW	placebo low dose
PI3K	phosphatidylinositol 3-kinase
P-MAPK	phosphorylated mitogen activated protein kinase
PPAR	peroxisome proliferator-activated receptor
PRK	protein kinase C-related kinase
PSS	physiological salt solution

PVDF	polyvinylidenedifluoride
SDBW	Sprague-Dawley buckwheat
SDC	Spague-Dawley control
SDS	sodium dodecyl sulfate
Ser	serine
SGLT	Na^+ dependent glucose transporter
SMC-PBS	smooth muscle cell phosphate-buffered saline
STAT	signal transducers and activators of transcription
STZ	streptozotocin
TBST	tris-buffered saline with tween
TCA	tricarboxylic acid cycle
TEMED	N, N, N', N' – Tetramethylethylenediamine
Thr	threonine
Tyr	tyrosine
TZD	thiazolidinediones

1. Introduction

Among metabolic diseases, diabetes mellitus is considered one of the most prevalent throughout the world. In diabetes, insufficient insulin action leads to metabolic disturbances in glucose homeostasis resulting in hyperglycemia. Prolonged hyperglycemia can result in serious complications including coronary heart disease, renal failure, blindness, limb amputations, and neurological problems (Knowler *et al*, 1995). In developed countries, diabetes and its complications place a significant burden on the economy and health care systems and is a leading cause of death (King *et al*, 1998; Dawson *et al*, 2002).

Insulin has an important role in the maintenance of blood glucose homeostasis. Inositol phosphoglycan (IPG) molecules containing either D-*chiro*-inositol (D-CI) or *myo*-inositol (MI) have been identified as putative mediators of insulin action based on their insulin-stimulated release and ability to mimic several actions of insulin (Field, 1997; Jones and Varela-Nieto, 1998, 1999). Differences in IPG activity and inositol metabolism have been observed in humans and animals with diabetes compared to nondiabetic controls (Suzuki *et al*, 1991; Sanchez-Arias *et al*, 1992; Sanchez-Gutierrez *et al*, 1994; Kennington *et al*, 1990; Asplin *et al*, 1993; Kunjara *et al*, 1999; Shaskin *et al*, 1997). In addition, urinary excretion of D-CI and MI has been reported as altered in diabetes, although there is disagreement on the direction of the change (Suzuki *et al*, 1991, 1994; Kennington *et al*, 1990; Ostlund *et al*, 1993). Nonetheless, a pattern of altered IPG activity and inositol metabolism has been observed in diabetes mellitus.

Interestingly, acute administration of synthetic D-CI ameliorates hyperglycemia in animal models of diabetes, presumably due to a correction of the altered metabolism of D-CI, however, the exact mechanism has not been determined (Ortmeyer *et al*, 1993; Fonteles *et al*, 2000). Clarification of the pattern of inositol excretion in diabetes and determining the mechanism for the antihyperglycemic effects of D-CI can contribute to the understanding of the pathogenesis of diabetes and may lead to novel treatments for the disease. Buckwheat contains relatively high levels of free D-CI and its derivatives known as fagopyritols and is a potential source of these compounds in the treatment of diabetes (Horbowicz and Obendorf, 1994). Buckwheat is produced in Canada, however, the majority of the crop is exported to Japan (Manitoba Agriculture and Food, 2003). The development of new uses for buckwheat and secondary processing activities can have implications for the buckwheat industry in Canada.

The following sections provide a review and general background information of areas relevant to this thesis which is focused on the pattern of urinary inositol excretion in diabetes and the potential antihyperglycemic and insulin-mimetic effects of a buckwheat extract.

2. Literature Review

2.1 Diabetes mellitus

Diabetes mellitus (DM) is a growing health problem in Canada, as in the rest of the world. The prevalence of diabetes in Canada was estimated to be 1.5 million people in 1995 and is expected to increase to 2.6 million Canadians by 2025 (King *et al*, 1998). The total economic burden of diabetes and its chronic complications in Canada for 1998 was estimated to be between $4.76 and $5.23 billion US dollars (Dawson *et al*, 2002).

DM is a chronic disease characterized by hyperglycemia, which is attributed to inadequate insulin action. Type 1 diabetes mellitus (DM-1), formerly known as insulin-dependent diabetes mellitus, is the result of autoimmune destruction of the pancreatic beta cells, which leads to a deficiency in insulin secretion (Jones and Varela-Nieto, 1999). Approximately 10 percent of people with diabetes have type 1. Individuals with DM-1 must administer insulin subcutaneously and self-blood glucose test up to several times a day to control hyperglycemia. Healthy eating and physical activity are also important in management of DM-1 (Health Canada, 1999).

Type 2 diabetes mellitus (DM-2), formerly known as non-insulin-dependent diabetes mellitus, is the most common form of diabetes. Approximately 90 percent of people with diabetes have type 2. Although it is most often diagnosed in adults, in recent years, more cases of DM-2 are being diagnosed in young people, especially those of Aboriginal descent (Health Canada, 1999). DM-2 is classified by normal or even increased insulin secretion

but reduced sensitivity of target cells (liver, muscle, fat tissues) to circulating insulin (Sherwood, 1997). Lifestyle changes such as diet and physical activity help control hyperglycemia in DM-2. Oral agents and/or insulin are required when hyperglycemia does not respond to diet alone (Ekoe, 1985).

2.1.1 Complications associated with diabetes mellitus

Chronic hyperglycemia is a major initiator of the complications arising from diabetes, which include microvascular complications, macrovascular complications, retinopathy and neuropathy. Microvascular complications of diabetes, also known as peripheral vascular disease, are caused by damage to the small blood vessels (Health Canada, 2002). This type of complication includes retinopathy and nephropathy. Diabetic retinopathy is a general term for all disorders of the retina caused by diabetes including glaucoma, cataracts and blindness (Health Canada, 2002). Nephropathy, or kidney disease, is a common and extremely serious result of diabetes. Macrovascular complications of diabetes, including cardiovascular disease, stroke, and lower limb amputations, are caused by the damage to large blood vessels that result from chronic hyperglycemia (Health Canada, 2002). Diabetic neuropathy is general term for a group of nerve diseases affecting the peripheral nerves. Complications that can arise from peripheral nerve damage include foot infections and ulceration, decreased sensation, increased sensitivity, muscle wasting and sexual dysfunction (Health Canada, 2002).

The damage to blood vessels and nerves leading to these complications occurs as a result of chronic hyperglycemia, increased production of advanced

glycation end products, and decreased blood circulation (Health Canada, 2002; Tirone and Brunicardi, 2001). Other mechanisms implicated in the complications arising from diabetes include activation of protein kinase C, stimulation of the polyol pathway and enhanced reactive oxygen species generation, increased vascular inflammation, altered gene expression of growth factors and cytokines, and platelet and macrophage activation (Yamagishi and Imaizumi, 2005; Obrosova and Julius, 2005). Thus, without proper management, diabetes can lead to serious complications including coronary heart disease, renal failure, blindness, limb amputations, neurological complications and premature death (Knowler *et al*, 1995). Overall, the risk for death among people with diabetes is about twice that of people without diabetes (Milo Ohr, 2002).

2.1.2 Regulation of glucose in the body

Humans require a constant source of energy, part of which is derived from the oxidation of glucose. When glucose is unavailable, most tissues can derive energy from amino acids and fatty acids, however, neuronal tissues lack the ability to metabolize ketone bodies (produced in the liver from fatty acids when glucose is unavailable) and cannot oxidize their own amino acids or fatty acids without undergoing adverse structural and functional changes. Thus, the ultimate purpose of glucose homeostasis is to provide free glucose to neuronal tissues. This is essential for survival as hypoglycemia leads to altered states of consciousness and can lead to death (Tirone and Brunicardi, 2001). Euglycemia is the result of a delicate balance between dietary glucose absorption, glucose uptake into tissues, glucose utilization for energy, storage of glucose as

glycogen, hepatic glucose output, and glucose reabsorption in the kidney (Kanzak and Pessin, 2001). The pancreas, liver, muscle, and brain have important roles in the body to sustain blood glucose levels within the normal range. Disruption of this delicate balance either through defects in pancreatic insulin secretion, liver glucose output, or peripheral tissue glucose uptake results in pathophysiological states of insulin resistance and diabetes (Kanzak and Pessin, 2001).

Glucose is obtained directly from the diet, following hydrolysis of ingested disaccharides and polysaccharides and is absorbed from the lumen of the small intestine. It is also synthesized from other substrates like amino acids via gluconeogenesis in organs such as the liver and kidney. Both dietary glucose and endogenously synthesized glucose must be transported from the circulation into target cells (Wood and Trayhurn, 2003). These processes involve the transfer of glucose across plasma membranes via integral transport proteins. There are two different types of transport proteins that enable the uptake of glucose into tissues: Na^+ dependent glucose transporters (SGLTs) and facilitative Na^+ independent glucose transporters (GLUTs) (Wood and Trayhurn, 2003; Zierler, 1999).

SGLTs utilize the Na^+-electrochemical gradient provided by the Na^+-K^+ adenosine triphosphate (ATP)ase pump to transport glucose into cells against a concentration gradient (Wood and Trayhurn, 2003). It has been reported that there are at least three isoforms of SGLTs although this family of glucose transporters is not as well characterized as compared to the GLUT family.

SGLTs are mostly known for their role in intestinal absorption and renal reabsorption of glucose. SGLT1 is present in the intestinal and renal epithelial cells. SGLT2 is found only in the epithelium of the kidney. Expression of SGLT3 was reported to be strong in liver, intestine, spleen, and muscle, and significantly lower in the kidney (Oku *et al*, 1999).

In contrast to the limited knowledge of SGLTs, the GLUTs have been comprehensively characterized in terms of structure, function and tissue distribution. GLUT1 is mainly expressed in the brain and erthyrocytes. Moderate levels of expression are also observed in adipose tissue, muscle and the liver. GLUT2 is expressed primarily in pancreatic beta cells, the liver and the kidneys. GLUT3 is present in tissues where the demand for glucose as a fuel is considerable, particularly the brain. GLUT4 is found in heart, skeletal muscle and adipose tissue and is also found in the brain. These facilitative transporters utilize the diffusion gradient of glucose across plasma membranes and exhibit different substrate specificities and kinetic properties. GLUTs 1-3 are insulin-independent glucose transporters whereas GLUT4 translocation is directly activated by insulin (Wood and Trayhurn, 2003).

Once glucose enters the cells, it is oxidized into energy via the Embden-Myerhof glycolytic pathway. Glycolysis occurs in the cytosol of all cells and results in the conversion of glucose to ATP, nicotinamide adenine dinucleotide (NADH) and pyruvate. ATP is utilized for cellular energy, whereas NADH and pyruvate provide substrates for other energy-producing reactions. When there is an adequate supply of oxygen to tissues, the overall reaction for the glycolytic

pathway ends in pyruvate, which is converted to acetyl CoA and enters the tricarboxylic acid (TCA) cycle where additional ATP is generated. Under anaerobic conditions such as prolonged exercise, the cells rely on glycolysis for ATP and pyruvate is converted to lactate. Conversion of pyruvate to lactate regenerates cofactors needed for glycolysis (Tirone and Brunicardi, 2001).

When energy needs are met, excess glucose can be stored as glycogen in the liver and muscle, however, the capacity to store glycogen in these tissues is limited. For example, a 70 kg person has approximately 70 g of liver glycogen and 400 g of muscle glycogen. When glycogen stores are filled, excess acetyl-CoA produced from glucose via glycolysis is converted to fatty acids (via lipogenesis) which are stored as triglycerides in unlimited amounts of adipose tissue. A 70 kg person has approximately 12 kg of triglyceride stored in adipose tissue, which could provide 2000 kcal/day for up to 2 months if necessary. When a need for glucose to provide energy or maintain euglycemia arises, the liver can release glucose from glycogen via glycogenolysis. The breakdown of glycogen stores from the liver results in a ready supply of glucose for the brain and other neural tissues that have an obligate need for free glucose. In contrast to the liver, muscle glycogen serves only as a local source of glucose, as muscle lacks the enzyme needed to release free glucose into circulation (Sherwood, 1997; Sizer and Whitney, 2003).

Besides glycogenolysis, glucose can also be produced from protein (via gluconeogenesis). However, excessive protein breakdown can lead to cell death and muscle wasting, so the body will shift away from the need for glucose to the

use of fat for energy in order to protect its protein supply. In addition to its abundance, fat is an efficient source of energy, providing 9 kcal/g compared to 4 kcal/g of glycogen or protein (Tirone and Bruncardi, 2001). Energy is produced from fat in the cell mitochondria via fatty acid oxidation. The acetyl-CoA produced by oxidation of fatty acids enters the TCA cycle to produce energy, however, the capacity of the TCA cycle to process the resulting acetyl-CoA decreases and thus, some acetyl-CoA is converted to acetoacetate, beta-hydroxybutyrate, and acetone, collectively called ketone bodies. Ketone bodies produced in the liver mitochondria are transported to other tissues such as brain, muscle or heart where they are converted back to acetyl-CoA to serve as an energy source. Although the brain normally uses only glucose for energy, during starvation ketone bodies can become the main energy source for the brain. In the metabolic condition called ketosis, ketone bodies are produced faster than they are consumed by tissues and the smell of acetone can be detected on a person's breath. This is one of many symptoms of poorly controlled diabetes mellitus (Sherwood, 1997; Sizer and Whitney, 2003).

2.1.3 The role of insulin in glucose homeostasis

Insulin is a pancreatic hormone, essential for achieving blood glucose homeostasis in mammals. The major function of insulin is to counter the action of hyperglycemia-generating hormones and sustain euglycemia (blood glucose concentrations within normal range of 4-6 mM) (King, 2002). Secretion of insulin from pancreatic beta cells is principally regulated by plasma glucose levels. In addition, plasma glucagon-like peptide-1 (GLP-1) and glucose-dependent

insulinotropic polypeptide (GIP) are released from the small intestine upon nutrient absorption, and stimulate insulin secretion from pancreatic beta cells (Enc *et al*, 2001).

The liver, because of its central role in glucose metabolism, is one of the major targets of insulin. Glucose uptake in the liver is via GLUT2, a low-affinity transporter that is not easily saturated at high plasma glucose levels. The liver and other tissues that utilize GLUT2 for glucose transport experience a rise in intracellular glucose when there is an increase in plasma glucose, which allows cells (hepatocytes and beta cells) to act as glucose sensors. In the pancreas, beta cells respond to the increased cellular glucose concentration by appropriately augmenting the secretion of insulin (Tirone and Brunicardi, 2001). In response to insulin, hepatic glucose uptake is dramatically augmented due to increased activity of enzymes involved in glucose utilization and storage via pathways of glycolysis and glycogenesis (King, 2002). For example, insulin increases expression of hepatic glucokinase, an enzyme that converts glucose to a form that is both trapped in the cell and capable of being further metabolized (Shimomura *et al*, 2000; King, 2002). In addition, insulin inhibits hepatic glucose output by decreasing the activity of key enzymes involved in gluconeogenesis and glycogenolysis (King, 2002). Hence, insulin maintains euglycemia by increasing hepatic glucose utilization and storage resulting in additional glucose uptake, and insulin suppresses hepatic glucose production and output.

Conversely, GLUT4 mediated uptake of glucose in muscle and adipose is directly responsive to insulin rather than plasma glucose concentrations. Among

the GLUT isoforms, only GLUT4 is insulin dependent. Insulin acts by stimulating the translocation of GLUT4-containing vesicles from intracellular stores to the plasma membrane resulting in an immediate 10-20 fold increase in glucose transport (Wood and Trayhurn, 2003). Thus, glucose uptake in skeletal muscle and adipose tissue is directly affected by insulin through its regulation of glucose transporters.

In addition to its role in regulating blood glucose metabolism, insulin stimulates lipogenesis, diminishes lipolysis, and increases protein synthesis. Insulin also modulates transcription, altering the cell content of numerous mRNAs, and stimulates growth, DNA synthesis, and cell replication (King, 2002).

2.1.4 Intracellular signaling pathways of insulin in the liver

The binding of insulin to its receptor in target tissues activates several distinct intracellular signaling pathways (Saltiel, 2001; Burks and White, 2001; Pessin and Saltiel, 2000; Summers *et al*, 1999). These events are essential for normal growth and development, and for normal homeostasis of glucose, fat and protein metabolism (Zick, 2004). The liver plays a central role in glucose metabolism via cell signaling pathways activated by insulin. Insulin binding to its receptor promotes the activation of its intrinsic tyrosine kinase activity and autophosphorylation. The activated insulin receptor catalyses the phosphorylation of several substrates, the most studied of these being the insulin receptor substrate (IRS) proteins (Saltiel, 2001; Burks and White, 2001; Pessin and Saltiel, 2000; Summers *et al*, 1999). Tyrosine-phosphorylated IRS then activates phosphatidylinositol 3-kinase (PI3K). PI3K has a major role in insulin

function, mainly via the activation of the Akt (also known as protein kinase B). Activated Akt induces glycogen synthesis, through inhibition of glycogen synthase kinase-3 (GSK-3). Promotion of fatty acid synthesis is also downstream of activated Akt whereas inhibition of cAMP-dependent protein kinase A (PKA) by Akt results in the downstream inhibition of lipolysis. In the mitochondria, activation of AMP-activated protein kinase (AMPK) (downstream from LKB1) inhibits fatty acid oxidation. Regulation of protein synthesis is also downstream from p70S6 kinase ($p70^{S6K}$) via phosphorylation of ribosomal protein S6. The Ras/mitogen activated protein kinase (MAPK) and Akt/mammalian target of rappamycin (mTOR) pathways both participate in this process (Saltiel, 2001; Burks and White, 2001; Pessin and Saltiel, 2000; Summers *et al*, 1999).

Insulin mediates transcription in the cell nucleus through the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, the Akt cascade, the protein kinase C-related kinase (PRK) pathway, as well as by activation of the Ras/MAPK pathway (Saltiel, 2001; Burks and White, 2001; Pessin and Saltiel, 2000; Summers *et al*, 1999). Regulation of various enzymes involved in glucose metabolism is mediated by insulin through regulation of transcription. For example, the activity of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme involved in hepatic gluconeogenesis is controlled primarily at the level of transcription, being inhibited by insulin (Alemzadeh, 2002).

2.1.5 Current approaches for treatment of diabetes mellitus

Treatment of DM depends on both the type of DM and the degree of disease progression. In DM-1, hyperglycemia results when pancreatic beta cells do not produce sufficient amounts of insulin. Insulin therapy for treatment of DM-1 involves subcutaneous administration of insulin and self-blood glucose testing up to several times a day. Available insulin preparations include human insulin (regular, neutral protamine Hagedorn or NPH, Lente, Ultralente) and insulin analogues (Lispro, Aspart, Glargine) which vary in their onset of action and duration (Dailey, 2004).

Unlike DM-1 where hyperglycemia is due to insufficient amounts of insulin, DM-2 is typically initiated by insulin resistance in target tissues. This results in continual hyperglycemia, hyperinsulinemia, a slow decline in compensatory measures, and eventually, a decrease in pancreatic beta cell function (Meriden, 2004). Thus, the treatment of DM-2 is dependent upon the stage or progression of the disease. Initially, an individual may respond to dietary changes and physical activity. If this is not effective for lowering hyperglycemia, oral agents may be used. The main options currently available for the treatment of DM-2 are alpha-glucosidase inhibitors, insulin secretagogues, biguanides, and thiazolidinediones (TZDs) (Meriden, 2004). Alpha-glucosidase inhibitors slow the digestion of carbohydrates, which delays glucose absorption and reduces postprandial elevations in plasma glucose levels. These agents, including acarbose and miglitol, treat hyperglycemia symptomatically rather than mechanistically. The insulin secretagogues lower plasma glucose levels by

stimulating pancreatic insulin release in response to a glucose load (Meriden, 2004). Among these are the sulphonylureas, which bind to a specific site on the ATP-sensitive potassium channels leading to their closure and the subsequent opening of calcium channels resulting in secretion of insulin (Rendell, 2004). Glibenclamide (glyburide), gliclazide, glipizide and glimepiride are the primary sulphonylureas in current clinical use for DM-2 (Rendell, 2004). The meglitinides are also insulin secretagogues that occupy the sulphonylurea receptor unit coupled to the ATP-sensitive potassium channel, but are not sulphonylureas (Rendell, 2004). Although insulin secretagogues are more effective than alpha-glucosidase inhibitors, they do not reverse insulin resistance (Meriden, 2004). Biguanides, including metformin, decrease hepatic glucose production and may enhance glucose uptake in muscle, but have minimal insulin-sensitizing effects (Rendell, 2004; Meriden, 2004). TZDs enhance sensitivity to insulin through activation of PPARγ and stimulate glucose uptake by muscle and adipose tissues. They directly affect the insulin signaling and response cascades, in addition to decreasing hepatic glucose production (Meriden, 2004). Among the TZDs are rosiglitazone and pioglitazone (Meriden, 2004).

No single agent appears capable of achieving target glucose levels in the majority of patients with DM-2. Combinations of agents are more successful in lowering glycosylated hemoglobin ($HbA1_c$) levels than a single agent (Rendell, 2004). In addition to these current therapies, novel approaches to treating diabetes are being investigated. An example is phlorizin, a natural product and dietary constituent found in a number of fruit trees (Ehrenkranz *et al*, 2004). This

plant flavonoid and its chemical analogues can produce renal glycosuria and block intestinal glucose absorption through inhibition of SGLTs. T-1095, a synthetic agent derived from phlorizin has been reported to increase urinary glucose excretion in diabetic animals, thereby decreasing blood glucose levels. Long term treatment with T-1095 reduced both blood glucose and $HbA1_c$ in diabetic rats and mice. Amelioration of hyperinsulinemia, hypertriglyceridemia, and microalbuminuria are among reported effects of this compound (Oku *et al*, 1999).

2.2 Inositol phosphoglycans as putative insulin mediators

New insight into the understanding of insulin action has emerged from the identification and partial characterization of two separate inositol phosphoglycan (IPG) molecules (Larner *et al*, 1988, 1989). In response to insulin, IPGs are hydrolyzed from glycosylphosphatidylinositols (GPIs) that are found in cell membranes. IPGs are considered putative insulin mediators based on their ability to mimic a large number of the metabolic actions of insulin both *in vitro* and *in vivo* (Field, 1997; Jones and Varela-Nieto, 1998, 1999). Although the structures of the IPGs have not been completely elucidated, one contains *myo*-inositol (MI) and glucosamine and the other contains D-*chiro*-inositol (D-CI) and galactosamine as core components (Jones and Varela-Nieto, 1999). Inositol is a hexahydroxycyclohexane that is structurally related to glucose. There are nine isomers of inositol that differ in their position of hydroxyl groups. MI is the most common occurring isomer in plants and animals whereas D-CI is relatively rare (Beemster *et al*, 2002). In addition to these core components, both types of IPG

also contain neutral sugars and phosphate residues (Jones and Varela-Nieto, 1999). The origin of the MI-containing IPG is thought to be MI-containing GPI, as both phospholipase C- and phospholipase D-mediated hydrolysis of GPI yield biologically active IPG molecules (Jones and Varela-Nieto, 1999). For the D-CI-containing IPG, the precursor and/or origin is unknown. The chemical structures of MI and D-CI are shown in Figure 2-1.

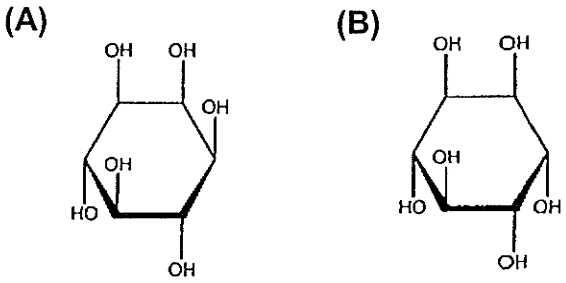

Figure 2-1. Chemical structures of (A) *myo*-inositol and (B) D-*chiro*-inositol.

Source: Kennington *et al*, 1990

2.2.1 Insulin-mimetic effects of IPGs on cell signaling and metabolism

The insulin-like activities of isolated IPGs and their chemically synthesized analogues have been widely investigated and have been previously reviewed (Field,1997; Jones and Varela-Nieto, 1998,1999). The MI-containing IPG has demonstrated *in vitro* stimulation of lipogenesis, glucose transport, glycogen synthesis, amino acid transport, protein synthesis, and GLUT-4 translocation. Stimulation of PI3K, MAPK and inhibition of GSK-3 by the MI-containing IPG

have also been documented. The MI-IPG was also able to regulate expression of PEPCK in rat hepatocytes (Alvarez *et al,* 1991).

Although it has been less studied, the D-CI-containing IPG has also demonstrated insulin mimetic effects *in vitro* including the activation of key protein phosphatases in pathways known to be stimulated by insulin. In particular, it has been demonstrated that the IPG containing D-CI activates pyruvate dehydrogenase (PDH) phosphatase and glycogen synthase phosphatase. These enzymes play a key role in the regulation of glucose disposal by oxidative metabolism (glycolysis) and by the non-oxidative route of storage by glycogen synthesis, respectively (Kunjara *et al*, 1999).

The insulin-mimetic effects of both IPGs have also been demonstrated *in vivo.* Both IPG subtypes increased glucose incorporation into diaphragm glycogen in normal rats and reduced hyperglycemia in streptozotocin (STZ) diabetic rats (Huang *et al*, 1993). Fonteles *et al* (1996) demonstrated that prolonged infusion of the D-CI-IPG was able to normalize plasma glucose in STZ rats to the same extent observed with insulin but without inducing hypoglycemia.

2.2.2 Altered IPG content and activity in diabetic animal models

The relationship between diabetes and GPI/IPG signaling is apparent by the differences in insulin-stimulated GPI hydrolysis in cells isolated from normal and diabetic rats. Insulin-induced generation of the D-CI-containing IPG was reduced in hepatocytes and adipocytes of diabetic Goto-Kakizaki (GK) rats compared to normal rats (Suzuki *et al*, 1991). GK rats are non-obese, mildly hyperglycemic (8.5 mmol/L), hyperinsulinemic, and have impaired intravenous

glucose tolerance due to a poor insulin secretory response (Portha *et al*, 1991). Impaired GPI-IPG dependent insulin-signaling systems have been reported in hepatocytes of STZ-induced diabetic rats (Sanchez-Arias *et al*, 1992) and obese fa/fa Zucker rats (Sanchez-Gutierrez *et al*, 1994). STZ rats are a model of DM-1, characterized by hyperglycemia and hypoinsulinemia whereas fa/fa Zucker rats (characterized by obesity, hyperinsulinemia, and impaired glucose tolerance) are a model for the early stages of DM-2. Sanchez-Arias *et al* (1992) reported that hepatocytes isolated from STZ rats had 60% less GPI, blocked hydrolysis of GPI in response to insulin, and markedly reduced uptake of IPG (40%), compared to control rats. A similar pattern of impaired GPI-dependent insulin signaling was demonstrated in fa/fa Zucker rats as hepatocyte content of GPI and IPG uptake were reduced by approximately 30% compared to lean control animals (Sanchez-Gutierrez *et al*, 1994).

2.2.3 Altered IPG content and activity in individuals with diabetes mellitus

Further evidence for the role of altered GPI/IPG systems in diabetes has been demonstrated in individuals with diabetes. According to Kennington *et al* (1990), D-CI was undetectable in IPGs from muscle-biopsy samples obtained from patients with type 2 diabetes whereas IPGs from normal subjects contained substantial amounts of D-CI. In addition, increased D-CI in IPGs from normal subjects but not in patients with DM-2 following insulin administration during euglycemic-hyperinsulinemic-clamp studies was also reported (Kennington *et al,* 1990).

Two biochemical assays are principally used to define the biological activity of the IPG sub-types. Activity of the MI-IPG is often measured by inhibition of PKA whereas stimulation of PDH phosphatase is used to determine the activity of the D-CI-IPG. Decreased activation of PDH phosphatase and reduced D-CI content were reported in the D-CI-containing IPG fractions isolated from urine, hemodialysate, and muscle of DM-2 subjects compared to control subjects whereas no difference in inhibition of PKA was observed in the MI-containing mediator (Asplin *et al* 1993). In contrast, Kunjara *et al* (1999) reported a 2-3 fold increase in the activation of lipogenesis in isolated adipocytes by the MI-containing mediator among DM-1 and DM-2 patients and no difference in activation of PDH phosphatase by the D-CI-containing mediator relative to control subjects. Shashkin *et al* (1997) reported that activation of PDH phosphatase by the D-CI-containing mediator in serum increased by 120% after glucose ingestion in healthy men whereas no change in bioactivity was observed among men with DM-2. Conversely, inhibition of PKA by the MI-containing mediator did not increase after glucose ingestion in either normal or DM-2 subjects.

2.2.4 Altered inositol metabolism in diabetes mellitus

Both MI and D-CI are found in mammalian tissues and cells as free inositols or as part of inositol phosphates and inositol phospholipids (Beemster *et al*, 2002; Pak *et al*, 1998). The incorporation of radioactive labeled MI and D-CI has been demonstrated in a wide range of tissues, with kidney being the highest (Pak *et al,* 1998). Besides dietary origin or oral administration, MI can be

endogenously synthesized from glucose in several tissues including the liver and kidney (Beemster *et al*, 2002). The conversion of MI to D-CI has also been demonstrated in various rat tissues including liver, muscle, adipose, and kidney although the exact mechanism is not known (Pak *et al*, 1998). In contrast, no or minimal conversion of D-CI to MI has been reported. The major product of MI breakdown is glucuronic acid with kidney as the main site of oxidation (Pak *et al*, 1998).

Experimental evidence suggests that inositol metabolism is altered in animal models of DM. Pak *et al* (1998) reported a difference in the metabolism of MI and D-CI in diabetic GK rats compared to normal Wistar rats. GK rats demonstrated increased incorporation of MI and D-CI into inositol phospholipids, and decreased incorporation of D-CI into inositol phosphates compared to normal rats. Decreased conversion of MI to D-CI in inositol phospholipids of insulin-sensitive tissues in GK rats was also reported.

2.2.5 Altered urinary inositol excretion in diabetes mellitus

In accordance with the altered inositol metabolism and GPI/IPG signaling defects, evidence suggests that urinary excretion of inositols is also altered in diabetes mellitus. While numerous rodent models of diabetes exist, there are few published reports available on their urinary inositol excretion. Suzuki *et al* (1991) reported elevated urinary MI excretion in diabetic GK rats, but reduced D-CI excretion compared to normal Wistar rats. Although a comment in the paper of Kennington *et al* (1990) indicated that the pattern of inositol excretion in STZ

and fa/fa Zucker rats is different from diabetic humans, no data to support this statement were provided.

Urinary excretion of free inositols appears to be altered in humans with diabetes mellitus compared to individuals with normal glucose tolerance (Kennington *et al,* 1990; Ostlund *et al*, 1993; Suzuki *et al*, 1994), however, there is disagreement regarding the direction of the change. Reduced urinary D-CI and elevated MI excretion have been reported in humans with DM-2 (Kennington *et al,* 1990; Suzuki *et al*, 1994) and impaired glucose tolerance (Suzuki *et al*, 1994). Kennington *et al* (1990) stated that analyses of urine from individuals with DM-1 revealed a wide variation in D-CI excretion, although no data were reported. In contrast, Ostlund *et al* (1993) found that individuals with either DM-1 or DM-2 had increased urinary D-CI and MI excretion compared with normal subjects. While it is apparent that urinary inositol excretion is altered in DM, the discrepancies between these reports have not yet been resolved. Furthermore, previous studies have employed extensive purification procedures to remove ionic substances, nitrogenous and peptide-like material prior to derivatization which may have contributed to losses of inositols.

2.2.6 Antihyperglycemic effects of free D-*chiro*-inositol

The effectiveness of chemically synthesized D-CI for lowering plasma glucose has been investigated in rats. A single dose of intragastric D-CI (2-15 mg/kg) administered to normal rats 2 hours before intraperitoneal glucose produced a 30-50% decrease in plasma glucose concentrations (Ortmeyer *et al*, 1993). In fed STZ rats, a single dose of intragastric D-CI (10 mg/kg) produced a

30-40% decrease in plasma glucose concentrations (Ortmeyer *et al*, 1993). Similarly, Fonteles *et al* (2000) also reported that a single dose of D-CI (15 mg/kg) attenuated elevated plasma glucose concentrations in STZ rats by 21% in 120 minutes.

Previous studies have evaluated the effectiveness of different doses of D-CI. Ortmeyer *et al* (1993) administered acute doses of 1-30 mg/kg in STZ rats and found maximal effects for plasma glucose lowering at 10 mg/kg. Fonteles *et al* (2000) reported significant plasma glucose lowering in STZ rats with a 15 mg/kg dose but not at 5 mg/kg. Although the antihyperglycemic effects of chemically synthesized free D-CI and IPGs containing D-CI have been demonstrated in diabetes, the exact mechanism for the glucose lowering effects of this compound has not yet been determined. Furthermore, the use of a natural dietary source of D-CI for management of diabetes has not been evaluated.

2.3 Buckwheat

Buckwheat a broadleaf crop that is botanically considered a fruit, although it is similar to cereals in its size, processing, and application characteristics. There are many species of buckwheat in the world, nine of which have agricultural meaning. Of the available species, however, common buckwheat (*Fagopyrum esculentum*) is widely grown whereas tartary buckwheat (*Fagopyrum tartaricum*) is only grown in some mountainous regions (Li and Zhang, 2001). Common buckwheat has been produced in Canada for over 40 years. Although buckwheat is grown in Ontario, Quebec, and sometimes

Saskatchewan, Manitoba is known as the "Buckwheat Capital of Canada" (Manitoba Agriculture and Food, 2003). The unique growing conditions in south central Manitoba make it one of the most productive places in Canada to grow buckwheat. The abundance of insects, such as honeybees, provides for greater success of the cross-pollination of the different flower types. The balanced fertility of the soil and lengthy growing season characteristic of the region ensure the proper growing environment for buckwheat. The presence of these conditions results in Manitoba producing over 70% of Canada's buckwheat crop (Manitoba Agriculture and Food, 2003).

Its edible portion, or groat, is a triangular kernel found inside the hull. The dehulled buckwheat groat is comprised of the testa or seed coat that encloses a large endosperm in which two thin folded cotyledons of the embryo are centrally located (Zheng *et al*, 1998). The majority, approximately 75%, of the buckwheat groat is the endosperm portion whereas the testa and embryo comprise about 25% of the groat (Li and Zhang, 2001). Buckwheat can be steamed, boiled, baked, or ground into flour and used in baked goods and noodles. It has been eaten for centuries in Japan, primarily in the form of soba noodles, a staple food. The majority of Canadian buckwheat is exported to Japan as the flavor and aroma of Manitoba buckwheat in particular meets the requirements of Japanese noodle makers. Since there is little demand for buckwheat locally, Manitoba produces it primarily for the export market (Manitoba Agriculture and Food, 2003). The development of new uses for buckwheat and secondary processing

activities can have economic implications for buckwheat growers in Manitoba and Canada.

2.3.1 Composition of buckwheat

Buckwheat is rich in nutrients and phytochemicals. The composition of dehulled common buckwheat is approximately 13% protein, 70% carbohydrate, 3% fat, 2% ash, and 12% moisture (Canadian Nutrient File, 2005; Zheng *et al*, 1998; Edwardson, 1996). Protein, lipids and soluble carbohydrates are concentrated in the embryo whereas starch is found in the endosperm of the buckwheat groat (Pomeranz and Robbins, 1972; Edwardson, 1996; Horbowicz *et al*, 1998; Obendorf et al, 2003). Buckwheat is relatively high in the essential amino acids leucine, lysine, phenylalanine, threonine, and valine, but is low in methionine and tryptophan. Of the non-essential amino acids, buckwheat contains high amounts of glutamic acid, aspartic acid and arginine (Li and Zhang, 2001; Canadian Nutrient File, 2005; Zheng *et al*, 1998). Buckwheat is very low in fat. Approximately 30-40% of the lipids in buckwheat are made up of the essential fatty acid linoleic acid and 30-45% are from oleic acid (Edwardson, 1996; Canadian Nutrient File, 2005). The lipid portion of buckwheat also contains plant sterols, largely made up of sitosterol (70%), as well as small amounts of stigmasterol, and campesterol (Obendorf *et al*, 1993; Udesky, 1988; Li and Zhang, 2001). Minerals reported to be present in buckwheat include potassium, magnesium, phosphorous, and iron (Udesky, 1988; Canadian Nutrient File, 2005; Li and Zhang, 2001; Wei *et al*, 2003). Small amounts of calcium, sodium, zinc, copper, manganese, and selenium have also been

reported (Canadian Nutrient File, 2005; Li and Zhang, 2001; Wei *et al*, 2003). Vitamins present in buckwheat include thiamin, niacin, riboflavin, vitamin B_6, and folate (Udesky, 1988; Canadian Nutrient File, 2005).

Starch is the major carbohydrate present in buckwheat and accounts for approximately 51-67% of the seed (Edwardson, 1996) whereas soluble carbohydrates comprise approximately 2% of the buckwheat groat (Horbowicz *et al*, 1998; Obendorf et al, 2003). Sucrose is the major (approximately 42%) soluble carbohydrate in buckwheat (Steadman *et al*, 2000), however, small amounts of glucose and fructose are also present (Obendorf *et al*, 1993). Buckwheat also contains fibre with amounts reported ranging from 4-7% (Canadian Nutrient File, 2005). Phytochemicals reported to be present in buckwheat include the flavonoids rutin, quercetin, catechins, and catechin oligomers. Reported flavonoid concentrations in common buckwheat varieties have been as high as 387 mg/g, 80 mg/g, and 21 mg/g for total flavonoids, rutin, and quercetin, respectively (Oomah and Mazza, 1996; Watanabe, 1998; Quettier-Deleu *et al*, 2000; Tian *et al*, 2002; Yokozawa *et al*, 2002; Fabjan *et al*, 2003).

2.3.2 D-*chiro*-inositol in buckwheat

Buckwheat seeds are relatively unique in that they contain the rare isomer D-CI and its galactosyl derivatives known as fagopyritols (Horbowicz and Obendorf, 1994). Most legumes accumulate flatulence-producing oligosaccharides (raffinose, stachyose, verbascose) in association with dessication tolerance (Horbowicz *et al*, 1998). Buckwheat does not accumulate

raffinose or stachyose, but the seeds tolerate dessication. Instead of the raffinose series oligosaccharides, buckwheat accumulates galactosyl cyclitols (sugar alcohols) (Horbowicz *et al*, 1998). Analysis of the soluble carbohydrate composition of buckwheat has revealed that a major soluble carbohydrate in buckwheat (40% of total) is Fagopyritol B1, named after the genus name *Fagopyrum*. The amounts of Fagopyritol B1 in buckwheat are similar to that of sucrose in buckwheat. The amounts of Fagopyritol B1 were reported to be 41 mg/g in the embryo portion of buckwheat (Horbowicz *et al*, 1998). Fagopyritol B1 is an alpha-galactosyl derivative of D-CI. Two different galactosyl *chiro*-inositol isomers, Fagopyritol A1 and B1, and two different di-galactosyl *chiro*-inositol isomers, Fagopyritol A2 and B2 are present in buckwheat. In addition, small quantities of Fagopyritol B3, a tri-galactosyl *chiro*-inositol, are present in buckwheat. These Fagopyritols are found primarily in the embryo portion of the buckwheat groat. Structures of the Fagopyritol B series are shown in Figure 2-2. Fagopyritols of the A series have a 1→3 linkage whereas those of the B series have a 1→2 linkage between galactopyranosyl and D-CI (Obendorf *et al*, 2000; Steadman *et al*, 2001).

Buckwheat is unique in that it contains five different fagopyritols whereas only one of the fagopyritols (B1) has been identified in soybean, lupine, lentil, and chickpea seeds (Horbowicz *et al*, 1998). Buckwheat also contains relatively high amounts of free D-CI and MI, as compared to other crops. D-CI has been detected in lupine, pigeon pea, soybean, chickpea and mungbean, however, only mungbean contains higher levels of free D-CI (mg/dry weight) than buckwheat.

Amounts of D-CI reported in buckwheat were 0.80 mg/g for the embryo portion (Horbowicz *et al*, 1998). Compositional differences exist between the common and tartary buckwheat species. In particular, the flavonoid content of tartary buckwheat is substantially higher than that of common buckwheat (Li and Zhang, 2001). In the present research, we used a common buckwheat variety Koto (*Fagopyrum esculentum* Moench), due to its availability and the presence of substantial amounts of D-CI and fagopyritols.

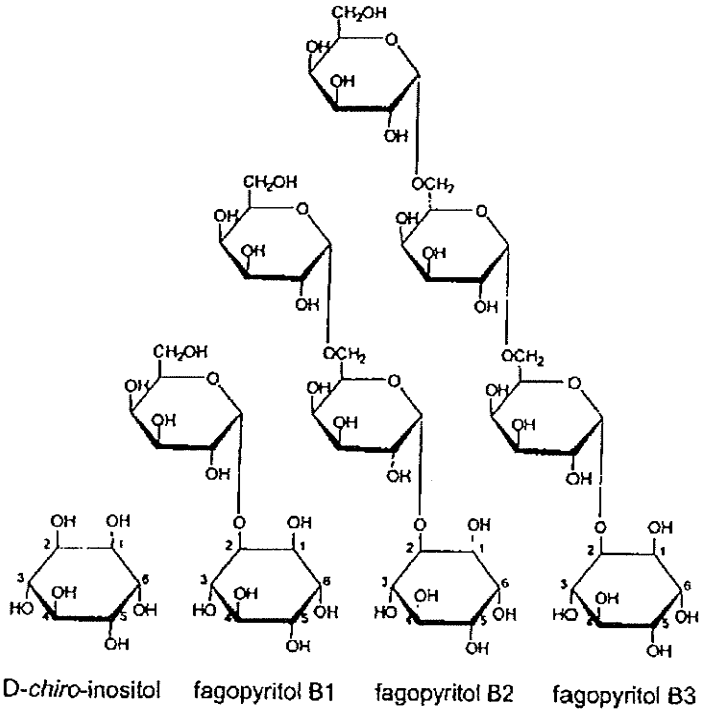

Figure 2-2. Chemical structures of the fagopyritol B series.

Source: Obendorf *et al*, 1997.

2.3.3 Antihyperglycemic effects of whole buckwheat

According to ancient folk-medicinal references, the health effects of buckwheat were intuited long ago when buckwheat was prescribed for high blood pressure, relaxation, relief and prevention of inflammation, excessive perspiration, nosebleed, and maintenance of good intestinal function (Udesky, 1988). More recently, buckwheat has attracted increasing attention for its potential in treatment of chronic diseases. Reported nutraceutical and functional food aspects of buckwheat include the hypocholesterolemic and anticarcinogenic effects of buckwheat proteins and the antioxidant activity of buckwheat flavonoids (Kayashita *et al*, 1995, 1997; Tomotake *et al*, 2000, 2001; Liu *et al*, 2001; Mukoda *et al*, 2001; Yokozawa *et al*, 2001, 2002).

The use of buckwheat in the management of diabetes mellitus has also been previously documented. Consumption of buckwheat as flour or biscuits made from buckwheat flour has demonstrated hypoglycemic effects (Lu *et al*, 1992; Wang *et al*, 1992; Bijlani *et al,* 1985). In patients with diabetes, Lu *et al* (1992) and Wang *et al* (1992) reported that consumption of biscuits containing 40% buckwheat flour 3 times per day for 30-45 days resulted in significant decreases in blood glucose concentrations. Bijlani *et al* (1985) also reported improved glucose tolerance and reduced fasting blood glucose after whole buckwheat flour supplementation in humans.

To date, the number of research studies investigating the antidiabetic properties of buckwheat is limited. Of the available studies, complete descriptions of study details and results are lacking. Furthermore, the active

components in buckwheat responsible for the observed effects are unknown. Previous studies reporting the hypocholesterolemic and anticarcinogenic effects of buckwheat have used isolated protein fractions and studies demonstrating the antioxidant activity have used flavonoid extracts (Kayashita *et al*, 1995, 1997; Tomotake *et al*, 2001, 2002; Liu *et al*, 2001; Yokozawa *et al*, 2001, 2002), however, the antihyperglycemic effects of a buckwheat extract containing D-CI and fagopyritols have not been evaluated. Although buckwheat appears to have potential as a functional food and/or nutraceutical in diabetes, further investigation is required to establish this relationship and clarify the contribution of individual compounds in buckwheat to the observed effects.

2.4.1 Animal models of diabetes mellitus

Several different rodent models are available for diabetes research. In animal models of DM-1, diabetes can either be induced by pharmacologic means or can spontaneously develop. There are several spontaneous models of DM-1, two of which have been extensively studied: the BBDP rat and the NOD mouse. The spontaneous development of DM-1 in these animal models is a result of autoimmune destruction of the pancreas beta cells (Sieher and Traystman, 1993). DM-1 can also be pharmacologically induced via a number of agents that selectively destroy pancreatic beta cells. STZ and alloxan are the most commonly used drugs (Sieher and Traystman, 1993).

The choice of animal model is usually dependent upon the aspect of disease pathogenesis or the complications to be studied and its manifestation in the animal model. For example, the BBDP rat has been useful in studying the

immunologic mechanisms of diabetes (Stiller *et al*, 1984) as well as diabetic neuropathy and retinopathy (Marliss *et al*, 1982) whereas models with STZ-induced DM-1 have been useful in studying the development of diabetic retinopathy (Engerman and Kern, 1987), nephropathy (Bloodworth and Engerman, 1980), and macrovascular complications (Engerman *et al*, 1977). Previous studies evaluating the effects of synthetic D-CI in an animal model of DM used STZ rats (Ortmeyer *et al*, 1993; Fonteles *et al*, 2000). In order to control for the possibility that negative results may be due to the choice of animal model, we used the same test conditions and animal model as in previous studies with synthetic D-CI to determine the efficacy of the BWC in the present research.

Numerous rodent models of DM-2 or the diabetes-obesity syndrome are available, and include spontaneously diabetic rodent models, genetically engineered transgenic and knockout rodent models, and artificially induced diabetic rodents. Genetically engineered models are particularly useful for studying the pathogenesis of diabetes and potential targets for developing novel treatment strategies. Artificially induced diabetic rodent models are useful in replicating the metabolic characteristics of human conditions and are more cost-effective than transgenic or knockout models. Spontaneously diabetic rodent models such as GK rats, fa/fa Zucker rats, db/db mice, and ob/ob mice are most commonly used in drug discovery (Chen and Wang, 2005). These animals vary in their metabolic characteristics, thus representing different aspects of DM-2.

The db/db mouse represents a model of DM-2, as these animals are characterized by obesity, hyperglycemia, hyperinsulinemia, and glucosuria, resulting from a single gene mutation (Coleman, 1982). The ob/ob mouse is similar to the db/db mouse in that it has marked obesity as a result of a single gene mutation, however, hyperglycemia and glucose intolerance in the ob/ob mouse occurs only in the early developmental states (Coleman, 1982). In addition, complications of diabetes such as retinopathies, peripheral neuropathies, and kidney lesions are not generally seen in ob/ob mice, whereas most of these complications have been described in the db/db mouse (Coleman, 1982). The fa/fa Zucker rat represents a model of impaired glucose tolerance, or the early stages of DM-2. These rats are obese due a specific mutation the leptin receptor, have hyperinsulinemia and impaired glucose tolerance, but are not hyperglycemic (Bray, 1977).

Previous studies in this laboratory have shown that dietary treatments have effectively ameliorated hyperglycemia in the female db/db mouse and glucose tolerance in the male fa/fa Zucker rat. In db/db mice, dietary zinc supplementation attenuated hyperglycemia and hyperinsulinemia (Simon and Taylor, 2001). In male fa/fa Zucker rats, feeding a dietary conjugated linoleic acid mixture improved oral glucose tolerance and reduced fatty liver (comment in Taylor and Zahradka, 2004). Since these animal models were previously used this laboratory, they were included the present research as models of DM-2 and IGT for characterization of the pattern of urinary inositol excretion as described in

Chapter 4, and for chronic feeding studies with a BWC as described in Appendix C.

2.4.2 The use of H4IIE cells for investigating insulin-stimulated signaling pathways

Cell culture studies can be useful for initial investigation of treatment mechanisms as they allow the study of a single aspect of a disease whereas an animal model represents a more complex system. Primary cell cultures are derived directly from excised animal tissues and cultured. The preparation of primary cultures is labor intensive and they can be maintained *in vitro* only for a limited period of time. Continuous cultures are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions or indefinitely. Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumor cells. Thus, this type of cell line has almost limitless availability and provides less variability in experimental conditions over time. H4IIE cells represent a continuous culture rat hepatoma cell line. The insulin-stimulated cell signaling pathways and insulin-stimulated glucose uptake in H4IIE cells has been well characterized in this laboratory (Zahradka *et al*, 1998, Yau *et al*, 1998; Yau *et al*, 1999), making this cell line ideal for evaluating the insulin-mimetic effects of the BWC in the present research.

3. Study Rationale

3.1 Urinary inositol excretion in diabetes mellitus

D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) have been identified as components of putative insulin mediators and impaired activity of these mediators has been reported in diabetes mellitus. Urinary excretion of free inositols also appears to be altered in diabetes mellitus, however, there is disagreement regarding the direction of the change. Reduced urinary D-CI and elevated MI excretion have been reported in humans with type 2 diabetes mellitus (DM-2) and impaired glucose tolerance (IGT) and an animal model of DM-2, whereas other reports suggest that both urinary D-CI and MI excretion is increased in individuals with DM-2. The discrepancies between these reports have not yet been resolved but may be due to different methods used for sample preparation. In addition, previous studies have used extensive purification steps which may have contributed to losses of these compounds.

To date, there is limited data on the pattern of inositol excretion in individuals with type 1 diabetes mellitus (DM-1). Furthermore, previous studies have included participants with DM-2 who were taking oral medications that may have affected inositol excretion. Animal models can be used for characterizing urinary inositol excretion in diabetes without confounding variables such as other medical conditions and the use of medications, however, there is a lack of data on urinary inositol excretion in the various diabetic animal models that are available. Thus, the purpose of the present research was to clarify the pattern of inositol excretion in animal models of DM-1, DM-2 and impaired glucose

tolerance (IGT) and in humans with DM-1 (treated with insulin therapy) and DM-2 (managed by diet only), using a method that does not require extensive purification of samples.

3.1.1 Hypothesis

Urinary excretion of D-CI and MI is elevated in humans with DM-1 and DM-2 and animal models of DM-1 and DM-2, characterized by hyperglycemia and glucosuria.

3.1.2 Objectives

Overall, the objective was to characterize the pattern of urinary inositol excretion in animal models of DM-1, DM-2 and IGT and in humans with DM-1 and DM-2.

Specific Objectives

1) To develop a method to analyze urinary inositols by gas chromatography using a specific silylation agent and that does not require extensive sample purification to avoid losses of D-CI and MI.
2) To determine the pattern of urinary inositol excretion in animal models of DM-1, DM-2, and IGT (STZ rats, db/db mice, fa/fa Zucker rats, respectively) compared to their nondiabetic counterparts.
3) To determine the pattern of urinary inositol excretion in humans with DM-1 (treated with insulin therapy) and DM-2 (managed by diet) compared to individuals without diabetes of the same gender, and similar age and body mass index.

3.2 Antihyperglycemic effects of buckwheat

In previous studies, administration of chemically synthesized D-*chiro*-inositol (D-CI) had an antihyperglycemic effect in STZ rats and normal rats given a glucose load, however, the mechanism for the observed effect was not determined. IPGs containing D-CI and *myo*-inositol (MI) are considered insulin-mimetics based on their ability to activate enzymes in insulin-related metabolic pathways, stimulate phosphorylation of cell signal transduction proteins known to be activated by insulin, and increase peripheral tissue glucose uptake. Buckwheat contains relatively high levels of D-CI and its galactosyl derivatives known as fagopyritols, and is a potential source of these compounds for the treatment of diabetes mellitus. Previous studies have used synthetic D-CI and the antihyperglycemic effects of a natural dietary source of D-CI have not been evaluated. The development of new uses for buckwheat can have implications for the buckwheat industry in Canada, which currently exports the majority of the crop to Japan. Furthermore, determining the mechanism for the antihyperglycemic effects of D-CI and the potential glucose-lowering effects of buckwheat, can contribute to the understanding of the pathogenesis of DM and the role of D-CI in the disease, and may lead to novel treatments. The liver plays a central role in glucose metabolism and H4IIE rat hepatoma cells provide a hepatic cell line where insulin is able to stimulate glucose metabolic pathways. Thus, the purpose of the present research was 1) to evaluate the use of buckwheat as a source of D-CI for lowering blood glucose concentrations under conditions from previous studies using chemically synthesized D-CI and 2) to evaluate the insulin-mimetic effects

of buckwheat on insulin-activated cell signaling pathways and cellular glucose uptake in H4IIE cells.

3.2.1 Hypothesis

An acute dose of a buckwheat concentrate (BWC), containing D-CI, will lower serum glucose concentrations in normal rats given a glucose load and in STZ rats.

The BWC, and D-CI, will have insulin-mimetic effects on activation of cell signal transduction proteins related to pathways involved in glucose metabolism and will stimulate glucose uptake in H4IIE cells.

3.2.2 Overall Objectives

- To determine the effects of an acute dose of a BWC, as a natural source of D-CI, on serum glucose concentrations in a diabetic animal model.
- To determine the *in vitro* effects of a BWC, and D-CI, on stimulation of cell signal transduction proteins related to glucose metabolism and known to be activated by insulin.
- To determine the *in vitro* effects of a BWC, and D-CI, on glucose uptake in H4IIE cells.

Specific objectives

1) To develop a processing technique to produce a concentrate from buckwheat that contains high levels of D-CI and can be tested for efficacy as a natural source of D-CI on glycemic control in diabetes mellitus.

2) To determine the effects of acute administration of a BWC on serum glucose concentrations of normal rats given a glucose load and of STZ rats under conditions used in previous studies with synthetic D-CI.
3) To establish the insulin-like equivalency of the BWC versus D-CI by investigating their effects on hepatic cell signal transduction proteins known to be stimulated by insulin and related to glucose metabolism using H4IIE rat hepatoma cells.
4) To characterize the intracellular signaling pathways activated by the BWC in relation to that of insulin in H4IIE cells.
5) To evaluate the effects of the BWC versus insulin and D-CI on glucose uptake using H4IIE cells.

4. Urinary D-*chiro*-inositol and *myo*-inositol excretion is elevated in the diabetic db/db mouse and streptozotocin-diabetic rat.

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Kawa J, Przybylski R, Taylor C. Urinary *chiro*-inositol and *myo*-inositol excretion is elevated in the diabetic db/db mouse and streptozotocin-diabetic rat.
Experimental Biology and Medicine. 2003. 228: 907-914.

4.1 Abstract

Inositol phosphoglycan molecules containing either D-*chiro*-inositol (D-CI) or *myo*-inositol (MI) have been isolated from various mammalian tissues and are putative mediators of insulin action. Urinary excretion of inositols appears to be altered in diabetes mellitus, however, the relationships with different types of diabetes are unclear. The objective of this study was to determine the urinary excretion of D-CI and MI in diabetic animal models, including streptozotocin (STZ) rats, db/db mice, and fa/fa Zucker rats. In STZ rats (Type 1 diabetes or DM-1), 12-hour urinary excretion of D-CI was elevated 336-fold and MI excretion was elevated 47-fold compared to their non-diabetic counterparts. When corrected for creatinine, D-CI excretion was 259-fold higher and MI excretion was 36-fold higher in STZ rats than in normal rats. The same pattern was observed in db/db mice (Type 2 diabetes or DM-2), where 12-hour urinary D-CI excretion was elevated 247-fold compared to normal mice. When corrected for creatinine, D-CI excretion was 2455-fold higher, and urinary MI excretion was elevated 8.5-fold in db/db mice compared to normal mice. The fa/fa Zucker rats (impaired glucose tolerance) had a pattern of urinary inositol excretion that was similar to the non-diabetic animals (lean Zucker rats, C57BL/6 mice and Sprague-Dawley rats). In summary, urinary D-CI and MI excretion was elevated in animal models of DM-1 and DM-2, concomitant with hyperglycemia and glucosuria.

4.2 Introduction

New insight into the understanding of insulin action has emerged from the identification and partial characterization of two separate inositol phosphoglycan (IPG) molecules (Larner *et al*, 1988; 1989). IPGs are hydrolyzed from glycosylphosphatidylinositols (GPIs) in cell membranes in response to insulin and are considered putative insulin mediators. Although the structures of the IPGs have not been completely elucidated, one contains MI and the other contains D-CI, a rare inositol isomer. Both MI and D-CI are incorporated into mammalian tissues and cells as free inositols or exist as inositol phosphates and inositol phospholipids (Beemster *et al*, 2002; Pak *et al*, 1998). GPIs found on the outer cell surface are derived from inositol phospholipids in cell membranes (Beemster *et al*, 2002). Besides dietary origin, MI can be synthesized from glucose and MI can be converted to D-CI (Beemster *et al*, 2002; Pak *et al*, 1992). Following GPI hydrolysis by phospholipases, IPGs are incorporated into the cell where they activate enzymes involved in glucose and lipid metabolism. The *in vitro* and *in vivo* insulin-like effects of both IPG mediators have been reviewed elsewhere (Varela-Nieto *et al*, 1996; Field, 1997; Jones and Varela-Nieto, 1998; 1999).

Experimental evidence suggests that IPGs are important in insulin-signaling and the pathogenesis of diabetes. Insulin-induced generation of the D-CI-containing IPG was reduced in hepatocytes and adipocytes of diabetic Goto-Kakizaki rats compared to normal rats (Suzuki *et al*, 1991). Impaired GPI-IPG dependent insulin-signaling systems have been reported in hepatocytes of streptozotocin (STZ)-induced diabetic rats (Sanchez-Arias *et al*, 1992) and obese

fa/fa Zucker rats (Sanchez-Gutierrez *et al*, 1994). Decreased bioactivity of the D-CI-containing IPG, measured by stimulation of pyruvate dehydrogenase phosphatase, has been documented in humans with type 2 diabetes (Asplin *et al*, 1993; Shaskin *et al*, 1997). Increased activity of the MI-containing mediator, determined by the stimulation of lipogenesis, has been described in individuals with type 1 or type 2 diabetes (DM-1 and DM-2, respectively) (Kunjara *et al*, 1999).

Urinary excretion of free inositols appears to be altered in diabetes mellitus compared to individuals with normal glucose tolerance (Kennington *et al*, 1990; Ostlund *et al*, 1993; Suzuki *et al*, 1994), however, there is disagreement regarding the direction of the change. Reduced urinary D-CI and elevated MI excretion have been reported in humans with DM-2 (Kennington *et al*, 1990; Suzuki *et al*, 1994) and impaired glucose tolerance (Suzuki *et al*, 1994), as well as in animal models of DM-2 (Suzuki *et al*, 1991; Ortmeyer *et al*, 1993). Kennington *et al* (1990) stated that analyses of urine from individuals with DM-1 revealed a wide variation in D-CI excretion. In contrast, Ostlund *et al* (1993) found that both DM-1 and DM-2 diabetic patients had increased urinary D-CI and MI excretion compared with normal subjects. The discrepancies between these reports have not yet been resolved. Ostlund *et al* (1993) reported urinary excretion of both D-*chiro*-inositol and L-*chiro*-inositol isomers, and the majority (>86%) of *chiro*-inositol was the D isomer.

The purpose of the present investigation was to determine the pattern of urinary inositol excretion in diabetic animal models. Twelve-hour urinary

excretion of MI and D-CI (the sum of D and L isomers) was analyzed in three different diabetic animal models and their non-diabetic counterparts. We chose STZ-induced diabetic rats (characterized by hyperglycemia and hypoinsulinemia) as a model of DM-1, db/db mice (characterized by hyperglycemia, hyperinsulinemia, and obesity) as a model of DM-2, and fa/fa Zucker rats (characterized by hyperinsulinemia and impaired glucose tolerance (IGT)) as a model for the early stages of DM-2.

4.3 Materials and Methods

Standards and chemical reagents

The *myo*-inositol standard, phenyl-α-D-glucoside (internal standard), trimethylsilylimidazole, pyridine, and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent alcohol was purchased from Fisher Scientific (Ontario, Canada). D-*chiro*-inositol standard was a gift from Dr. S.G. Angyal (University of New South Wales, Australia).

Animals and diet

A protocol for animal care procedures was approved by the University of Manitoba Protocol Management and Review Committee. Animals were maintained in a controlled environment of 21-23°C, 55% humidity, and a 14 hour light, 10 hour dark cycle.

Type 1 diabetes mellitus (DM-1)

Six week old male Sprague-Dawley rats were obtained from Central Animal Holding, Winnipeg, Manitoba and were maintained on standard laboratory chow (Prolab RMH 3000, Purina Mills, Richmond, Indiana). Following a one week acclimatization period, diabetes was induced by intraperitoneal injections of 60 mg STZ/kg body weight/day on two consecutive days. Three days post-injection, hyperglycemia was confirmed by measuring serum glucose. Urine and blood samples were obtained from control animals (Sprague-Dawley rats) and STZ rats one week post-injection.

Type 2 diabetes mellitus (DM-2)

Four week old female db/db mice (Jackson Laboratories, Bar Harbor, Maine) were maintained on a semi-purified diet based on the AIN-93G formulation for 7 weeks (Lepage *et al*, 1999). Control mice were female C57BL/6 mice (Central Animal Holding, Winnipeg, Manitoba) fed standard laboratory chow (Prolab RMH 3000, Purina Mills, Richmond, Indiana).

Impaired glucose tolerance (IGT)

Five week old male fa/fa and lean Zucker rats (Charles River Laboratories, Wilmington, MA) were maintained on a semi-purified diet based on the AIN-93G formulation for 3 weeks (Lepage *et al*, 1999). To confirm IGT in fa/fa compared to lean Zucker rats, an oral glucose tolerance test (OGTT) was administered following a 5 hour fast. For the OGTT, blood samples were collected from the saphenous vein immediately prior and 15, 30, and 60 minutes following an oral dose of a 70% glucose solution (1 g glucose/kg body weight).

Blood collection

Blood samples were obtained from animals following a 12-hour fast with the exception of STZ rats, which were in the fed state. A blood sample was collected via the saphenous vein from fa/fa and lean Zucker and STZ rats. Trunk blood was collected from Sprague-Dawley rats, C57BL/6 and db/db mice following termination by CO_2 asphixyation and decapitation. Blood samples were stored on ice until centrifuged to obtain serum. Serum was stored at -20°C until analyzed.

Urine collection

Twelve-hour urine specimens were collected during an overnight fast in polycarbonate metabolic cages (Nalgene, Fisher Scientific), except that urine from STZ rats was collected during the day. During urine collection, animals were given free access to water, but not feed, to ensure that urine samples were not contaminated by the diets. The volume of each 12-hour urine sample was calculated using weight and aliquots were stored at -20°C until analyzed.

Preparation of urine for inositol analysis

The D-CI and MI content was determined in 12-hour urine specimens collected from C57BL/6 and db/db mice, lean and fa/fa Zucker rats, Sprague-Dawley and STZ-treated rats. One volume of ethanol was added to an equal volume of urine (0.1-0.5 mL). Samples were vortexed and evaporated to dryness under nitrogen at 40°C. Dried samples were sonicated for 5 minutes with 1 mL of trimethylsilylmidazole:pyridine (1:1, v/v) which contained 200 μg phenyl-α-D-glucoside as an internal standard, and derivatized for 1 hour at 80°C.

Preparation of diet samples for inositol analysis

The D-CI and MI content was determined in the diets according to a modification of the method used for urine samples. Briefly, 1 gram of ground diet sample was thoroughly homogenized with 5 mL of ethanol:water (1:1, v/v). The homogenate was vacuum filtered and the remaining residue re-extracted with the same volume of solvent. An aliquot of the combined filtrates was evaporated and derivatized as described for urine specimens.

Inositol analysis by gas chromatography

Two μL of derivatized samples or standards were injected into a Shimadzu gas chromatograph model GC-17A (Columbia, Maryland) equipped with a flame ionization detector and split injector. Inositols were separated on a RTX-5MS capillary column (25 m length, 0.25 mm ID, and 0.25 μm film thickness; Restek, Bellefonte, PA). Column temperature was programmed from 150°C to 200°C at the rate of 3°C/min, then to 325°C at the rate of 7°C/min. Initial and final temperatures were held for 5 and 20 minutes, respectively. The injector and detector temperatures were held at 270°C and 350°C, respectively. The carrier gas was hydrogen at 1.5 mL/min while the split ratio used was 1:40. Inositols were quantified using phenyl-α-D-glucoside as the internal standard. Standard curves for D-CI and MI were linear from 1 to 100 μg with r^2 values of 0.999 and an average error of 3.18% and 3.68%, respectively. D-CI standard was added to selected samples to confirm peak identification. Urine and diet samples were analyzed in duplicate.

Determination of recovery and detection limits for inositols

Recovery was determined using two levels (1.4 and 20 μg) of D-CI and MI standards added to 0.5 mL aliquots of rat urine. To evaluate the recovery, total D-CI and MI was calculated for each sample and compared to a urine aliquot without added inositol standards. Three repetitions were done at each level. The detection limit for D-CI and MI was defined when peaks of these inositols were no longer detected in decreasing concentrations of rat urine aliquots (0.1 mL to 0.005 mL).

Biochemical analyses

Creatinine in urine was analyzed in duplicate using a colorimetric assay (Procedure #555, Sigma Chem. Co., St. Louis, MO). Glucose in the serum and urine was assessed in triplicate using an enzymatic colorimetric kit (Procedure # 315, Sigma Chem. Co., St. Louis, MO). Insulin in the serum was analyzed in duplicate using a Sensitive Rat Radioimmunoassay kit (Linco, St. Charles, MO). Method details are provided in Appendix A.

Statistical analysis

Statistical significance between each set of diabetic rats and their normal counterpart was determined by Student's *t* test (SAS v.8.2, SAS Institute Inc., Cary, NC). Time course data was analyzed by ANOVA and by Duncan's multiple range test for means testing. Correlations were analyzed using Pearson's Correlation Coefficient. Differences were accepted as significant at $p<0.05$. Data are expressed as the mean ± SE.

4.4 Results

Recovery and detection limit of inositols

Recovery of 1.4 μg D-CI and MI added to 0.5 mL of urine was 98.2 ± 3.0% and 101± 4%, respectively. Recovery of 20 μg D-CI and MI added to 0.5 mL of urine was 88.9 ± 3.5% and 83.0 ± 1.1%, respectively. For the recovery experiment, samples were done in triplicate. The detection limit for D-CI and MI was 2 ng or 1 μg/mL urine. Typical chromatograms from a diabetic STZ rat, normal Sprague-Dawley rat and inositol standards are shown in Figure 4-1.

Pattern of inositol excretion in STZ rats (DM-1)

Administration of STZ to normal rats destroys insulin-producing beta cells of the pancreas resulting in hypoinsulinemia and hyperglycemia. In this study, STZ-diabetic rats had hyperglycemia and glucosuria compared to their normal counterparts (Table 4-1). Urinary D-CI excretion was elevated 336-fold in STZ rats compared to their non-diabetic counterparts (Table 4-1). Excretion of MI in STZ rats was also 47-fold higher than in normal rats (Table 4-1). When expressed per creatinine, D-CI excretion was elevated 259-fold and MI was 36-fold higher in STZ rats compared to normal rats (Table 4-1 and Figure 4-2A). STZ rats excreted more D-CI than MI whereas the reverse was observed in normal rats. As a result, the ratio of MI/D-CI was 7.4-fold lower in STZ rats compared to non-diabetic rats (Table 4-1 and Figure 4-3).

We also examined the relationships between urinary excretion of inositols and diabetic indices. In STZ rats, urine glucose was positively correlated with 12-hour excretion of both D-CI (Figure 4-4) and MI ($r = 0.95$, $p \leq 0.0001$; results

not shown). When corrected for creatinine, both urinary excretion of D-CI and MI were also positively correlated with urine glucose ($r = 0.73$, $p<0.05$ and $r = 0.82$, $p<0.01$, respectively). No significant relationships were observed between urinary excretion of inositols and diabetic indices in normal Sprague-Dawley rats.

Pattern of inositol excretion in db/db mice (DM-2)

Diabetic db/db mice had hyperglycemia, hyperinsulinemia, and glucosuria (Table 4-2). In db/db mice, urinary D-CI excretion was elevated 247-fold compared to their non-diabetic counterparts (Table 4-2). When expressed per creatinine, urinary D-CI in db/db mice was 2455-fold higher than in normal mice (Table 4-2 and Figure 4-2B), despite lower creatinine excretion in db/db mice (Table 4-2). Urinary MI excretion of db/db mice was also elevated 8.5-fold compared to normal mice when corrected for creatinine (Table 4-2 and Figure 4-2B). The db/db mice excreted more D-CI than MI whereas the reverse was observed in non-diabetic mice. As a result, the ratio of MI/D-CI was 175-fold lower in db/db mice compared to normal mice (Table 4-2 and Figure 4-3).

A comparison of the urinary inositol excretion of db/db mice at 5, 8, and 11 weeks of age showed a significant increase in D-CI excretion (Figure 4-5). There was an 11-fold increase in D-CI excretion by 8 weeks and a 21-fold increase at 11 weeks compared to D-CI excretion at 5 weeks as expressed per creatinine. Furthermore, urinary D-CI excretion of db/db mice was higher than normal mice at 8 and 11 weeks of age ($p<0.05$ and $p<0.005$, respectively). When expressed per 12 hours, the same pattern of increasing D-CI excretion was observed in db/db mice where urinary D-CI was 58.2 ± 58.2 nmol at 5 weeks of age, 437 ±

117 nmol at 8 weeks of age, and 2820 ± 1030 nmol at 11 weeks of age. These values were significantly higher than in normal mice where 12-hour D-CI excretion was 3.26 ± 1.99 nmol at 8 weeks of age and 11.4 ± 4.3 nmol at 11 weeks of age ($p<0.05$). The increase in D-CI excretion in db/db mice was concurrent with an increase in urine glucose. Twelve-hour urinary glucose excretion in db/db mice was 10.9 ± 4.9 μmol at 5 weeks, 176 ± 37μmol at 8 weeks, and 457 ± 115 μmol at 11 weeks. No difference in 12-hour urinary glucose excretion was observed in normal mice at 8 and 11 weeks with values of 0.04 ± 0.01 μmol and 0.11 ± 0.03 μmol, respectively. Furthermore, urine glucose excretion was significantly lower in normal mice compared to db/db mice at 8 weeks of age (0.11 ± 0.07 vs. 176 ± 37; $p<0.01$) and 11 weeks of age (Table 4-2). This indicates that the increase in D-CI excretion is related to the increasing severity of diabetes, as indicated by glucosuria, rather than an effect of age.

We also examined the relationships between urinary excretion of inositols and diabetic indices in normal and diabetic mice. In db/db mice, 12-hour urinary D-CI excretion was positively correlated with urine glucose ($r = 0.83$, $p<0.05$). When corrected for creatinine, MI excretion was elevated compared to normal mice (Table 4-2 and Figure 4-2B) and was positively correlated with serum glucose ($r = 0.88$, $p<0.05$). No significant relationships were observed in normal mice.

Pattern of inositol excretion in fa/fa Zucker rats (IGT)

The fa/fa Zucker rats were hyperinsulinemic (Table 4-3) and had impaired glucose tolerance during an oral glucose tolerance test when compared to lean rats (Figure 4-6). Diabetes is defined as a fasting serum glucose concentration greater than 7 mmol/L (Meltzer *et al*, 1998). Although fasting serum glucose concentrations of fa/fa rats were significantly higher than lean rats (Table 4-3, 6.66 ± 0.30 vs. 4.32 ± 0.36, $p<0.005$), fa/fa rats were not hyperglycemic according to the definition of diabetes. Urinary glucose excretion was 1.4-fold higher in fa/fa rats compared to lean rats (Table 4-3). However, fa/fa rats did not have glucosuria when compared to the db/db mice and STZ rats where urine glucose excretion was elevated 4155- and 15528-fold, respectively (Table 4-1 and Table 4-2). There was no significant difference in the urinary D-CI or MI excretion between fa/fa and lean rats (Table 4-3 and Figure 4-2C). There was also no difference in the ratio of MI/D-CI between lean and fa/fa rats (Table 4-3 and Figure 4-3). Similar to the normal Sprague-Dawley rats and normal C57BL/6 mice, both lean and fa/fa rats excreted more MI than D-CI (Table 4-3, Figure 4-2C). In fa/fa Zucker rats, 12-hour urinary D-CI excretion was positively correlated with urine glucose ($r = 0.97$, $p<0.05$), however, no significant relationships were demonstrated between excretion of urine inositols and diabetic indices in lean rats.

Table 4-1. Characteristics of STZ rats (DM-1 animal model) compared to control counterparts[1].

	Normal[2]	DM-1[3]
Serum glucose (mmol/L)	6.12 ± 0.32	30.4 ± 0.9****
Urine glucose (μmol/12 hours)	1.23 ± 0.15	19100 ± 2850***
Urine creatinine (μmol/12 hours)	28.4 ± 3.3	38.4 ± 3.7
Urine D-*chiro*-inositol		
(nmol/12 hours)	120 ± 14	40300 ± 7340***
(μmol/L / mmol/L creatinine)	4.41 ± 0.63	1140 ± 274**
Urine *myo*-inositol		
(nmol/12 hours)	209 ± 12	9850 ± 1790***
(μmol/L / mmol/L creatinine)	7.69 ± 0.84	273 ± 62**
Ratio *myo*-inositol / D-*chiro*-inositol	1.86 ± 0.29	0.25 ± 0.03**

[1]Values are means ± SE; * $p<0.05$, ** $p<0.005$, *** $p<0.001$, ****$p<0.0001$ as determined by t-test.
[2] n=5 for 7 week old Sprague-Dawley rats; mean body weight = 185 ± 2 g
[3] n=9 for 8 week old STZ rats; mean body weight = 272 ± 4 g

Table 4-2. Characteristics of db/db mice (DM-2 animal model) compared to control counterparts[1].

	Normal[2]	DM-2[3]
Serum glucose (mmol/L)	8.25 ± 0.49	40.6 ± 2.3***
Serum insulin (ng/mL)	0.15 ± 0.05	2.59 ± 0.28**
Urine glucose (μmol/12 hours)	0.11 ± 0.03	457 ± 115*
Urine creatinine (μmol/12 hours)	4.74 ± 0.98	0.57 ± 0.15*
Urine D-*chiro*-inositol		
(nmol/12 hours)	11.4 ± 4.3	2820 ± 1030*
(μmol/L / mmol/L creatinine)	1.87 ± 0.68	4590 ± 797**
Urine *myo*-inositol		
(nmol/12 hours)	106 ± 21	117 ± 40
(μmol/L / mmol/L creatinine)	23.0 ± 1.4	196 ± 28**
Ratio *myo*-inositol / D-*chiro*-inositol	7.00 ± 1.13	0.04 ± 0.00**

[1]Values are means ± SE; * $p<0.05$, **$p<0.003$, *** $p<0.0001$ as determined by t-test.

[2] n=6 for 11 week old C57BL/6 mice; mean body weight = 18 ± 1 g

[3] n=6 for 11 week old db/db mice; mean body weight = 31 ± 1 g

Table 4-3. Characteristics of fa/fa Zucker rats (IGT animal model) compared to control counterparts[1].

	Normal[2]	IGT[3]
Serum glucose (mmol/L)	4.32 ± 0.36	6.66 ± 0.30**
Serum insulin (ng/mL)	0.47 ± 0.04	7.33 ± 0.70**
Urine glucose (μmol/12 hours)	0.86 ± 0.07	1.21 ± 1.13*
Urine creatinine (μmol/12 hours)	32.7 ± 1.7	31.6 ± 4.2
Urine D-*chiro*-inositol		
(nmol/12 hours)	78.6 ± 24.3	72.3 ± 7.0
(μmol/L / mmol/L creatinine)	2.52 ± 0.81	2.32 ± 0.10
Urine *myo*-inositol		
(nmol/12 hours)	314 ± 49	372 ± 57
(μmol/L / mmol/L creatinine)	9.79 ± 1.82	11.92 ± 1.65
Ratio *myo*-inositol / D-*chiro*-inositol	3.55 ± 0.54	5.21 ± 0.84

[1]Values are means ± SE; *p<0.05, ** p<0.003 as determined by the t-test.
[2] n=5 for 8 week old lean Zucker rats; mean body weight = 255 ± 9 g
[3] n=4 for 8 week old fa/fa Zucker rats; mean body weight = 414 ± 13 g

Table 4-4. Pattern of diabetic indices and urinary excretion of inositols in animal models of type 1 and type 2 diabetes mellitus and impaired glucose tolerance compared to their control counterparts[1].

	DM-1	DM-2	IGT
Hyperglycemia	✓	✓	
Hyperinsulinemia		✓	✓
Hypoinsulinemia	✓		
Glucosuria	✓	✓	
Elevated urinary D-*chiro*-inositol[2]	✓	✓	
Elevated urinary *myo*-inositol[2]	✓	✓	
Reduced *myo*-inositol / D-*chiro*-inositol	✓	✓	

[1] STZ rats for model of type 1 diabetes mellitus (DM-1) vs. Sprague-Dawley rats; db/db mice for model of type 2 diabetes mellitus (DM-2) vs. C57BL/6 mice; fa/fa Zucker rats for model of impaired glucose tolerance (IGT) vs. lean Zucker rats
[2]Expressed as μmol/L / mmol/L creatinine

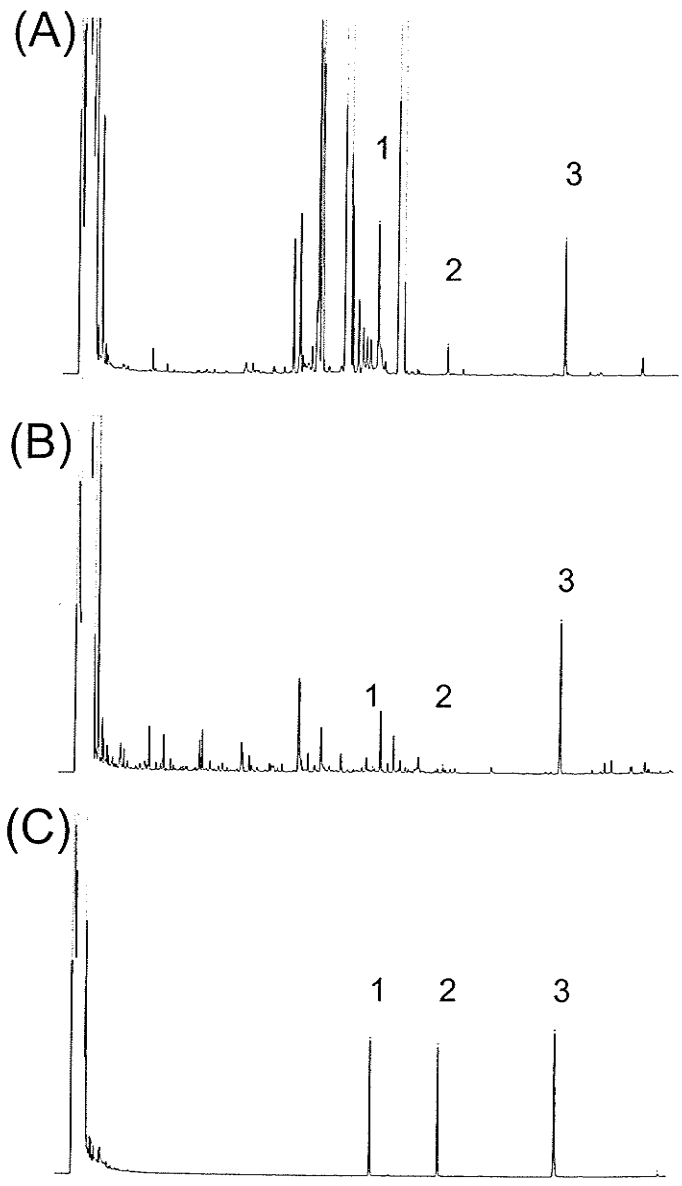


Figure 4-1. Chromatograms of silylated components in urine of (A) Streptozotocin rat, and (B) Sprague-Dawley rat, and the standard (C). 1=D-*chiro*-inositol; 2=*myo*-inositol; 3=internal standard (phenyl-α-D-glucoside).

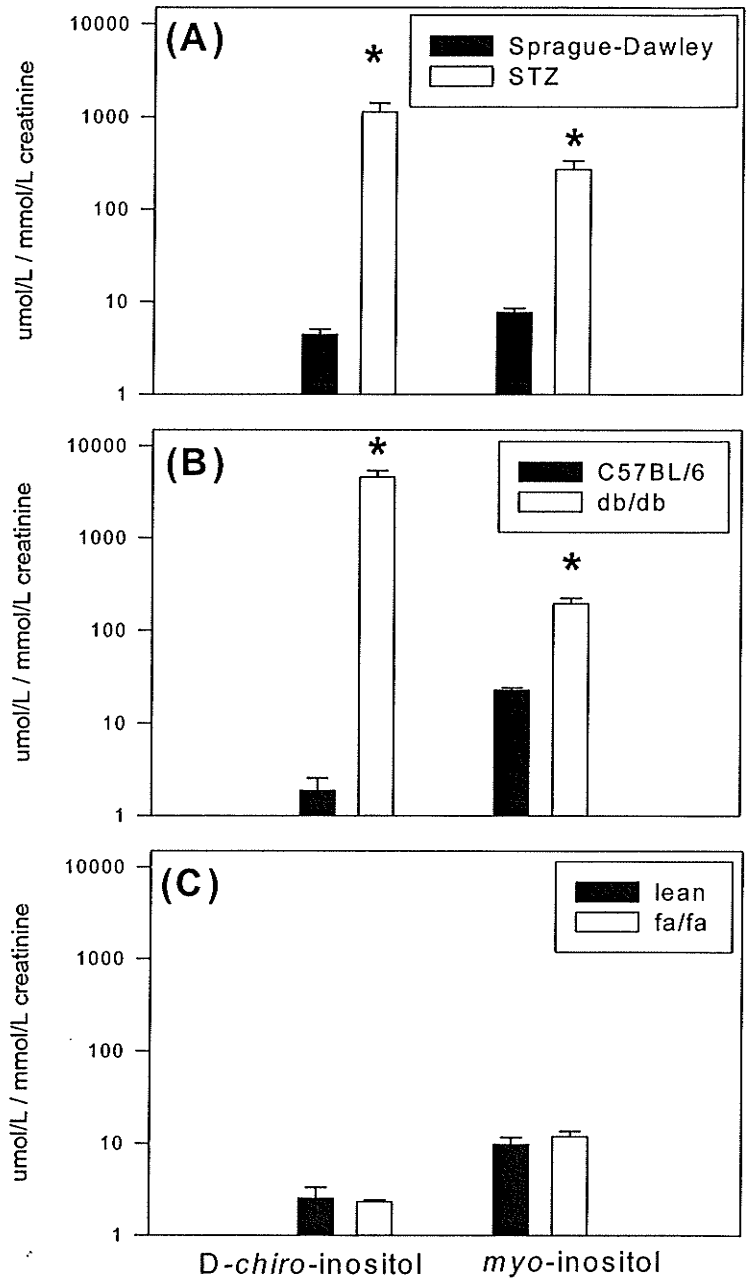


Figure 4-2. Urinary inositol excretion in (A) Sprague-Dawley and streptozotocin-induced diabetic rats, (B) C57BL/6 and db/db mice, and (C) lean and fa/fa Zucker rats. An * indicates differences ($p<0.005$) in D-*chiro*-inositol and *myo*-inositol excretion for diabetic animals vs. their normal counterparts.

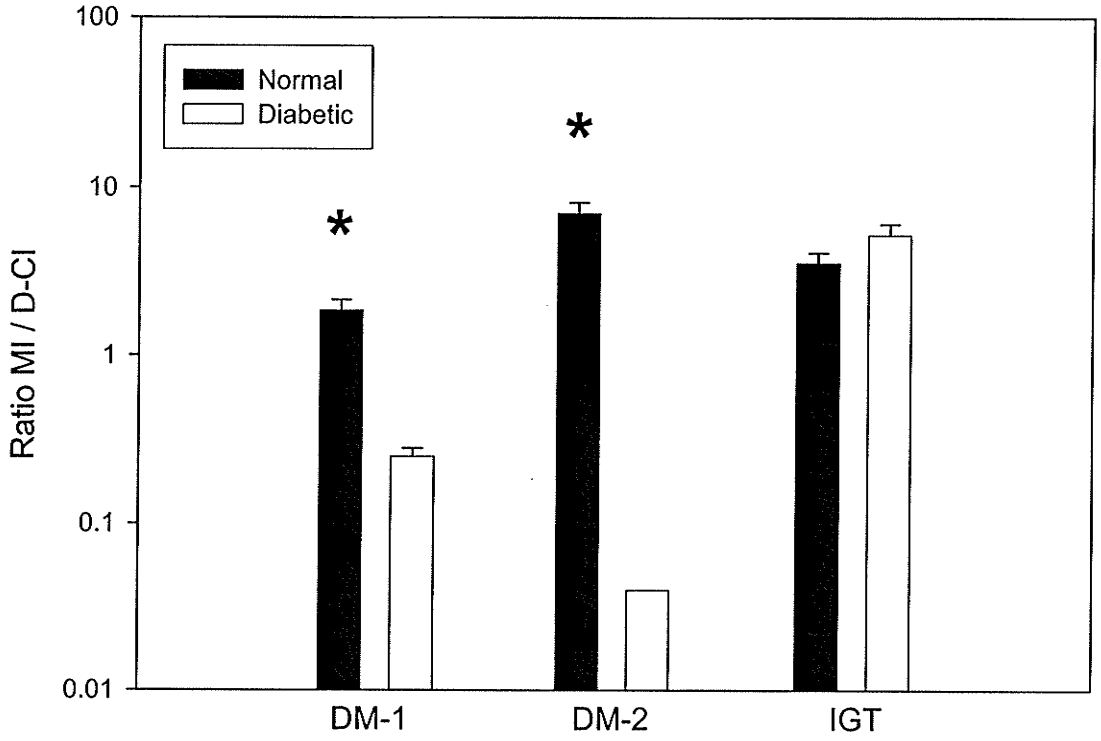


Figure 4-3. Ratio of *myo*-inositol (MI)/ D-*chiro*-inositol (D-CI) excretion in normal and diabetic animal models. DM-1 normal=Sprague-Dawley rats, DM-1 diabetic=STZ rats; DM-2 normal=C57BL/6 mice, DM-2 diabetic=db/db mice; IGT normal=lean Zucker rats, IGT diabetic=fa/fa Zucker rats. An * indicates differences ($p<0.005$) in the ratio of urinary MI/D-CI for diabetic animals vs. their normal counterparts.

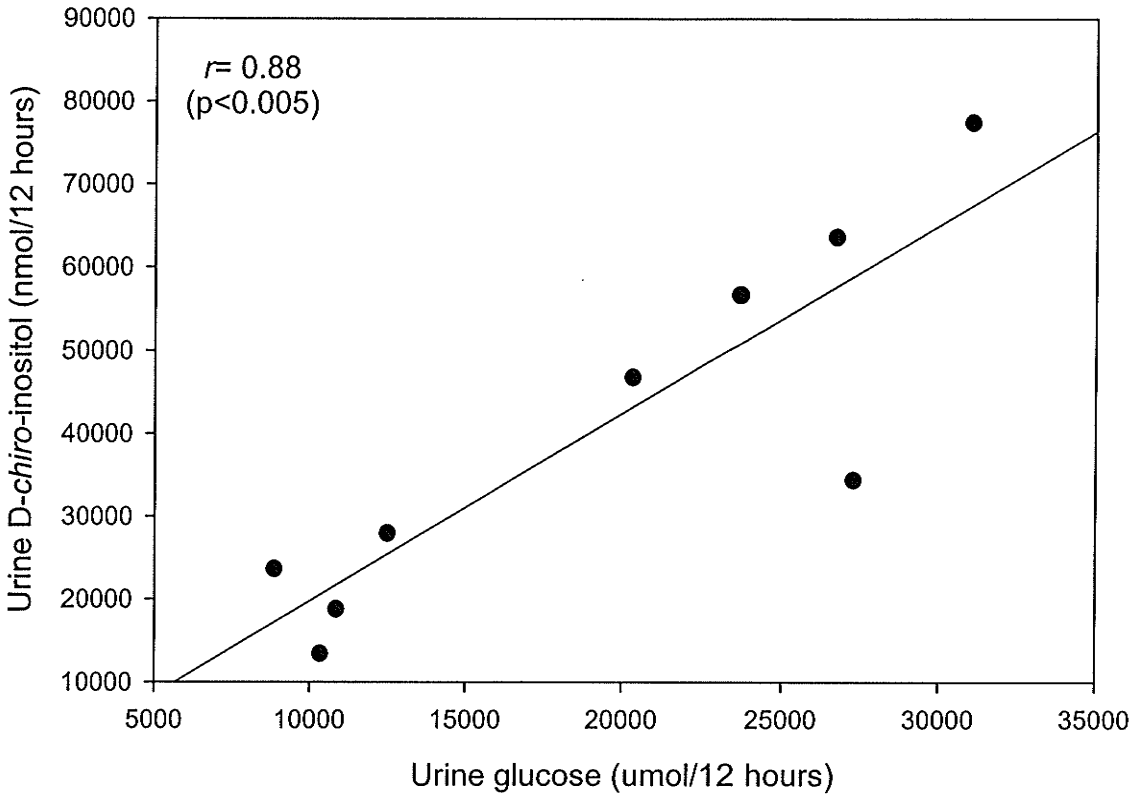


Figure 4-4. Scatter plot of urine glucose excretion versus urine D-*chiro*-inositol excretion in STZ rats. Data points are the values obtained from individual animals.

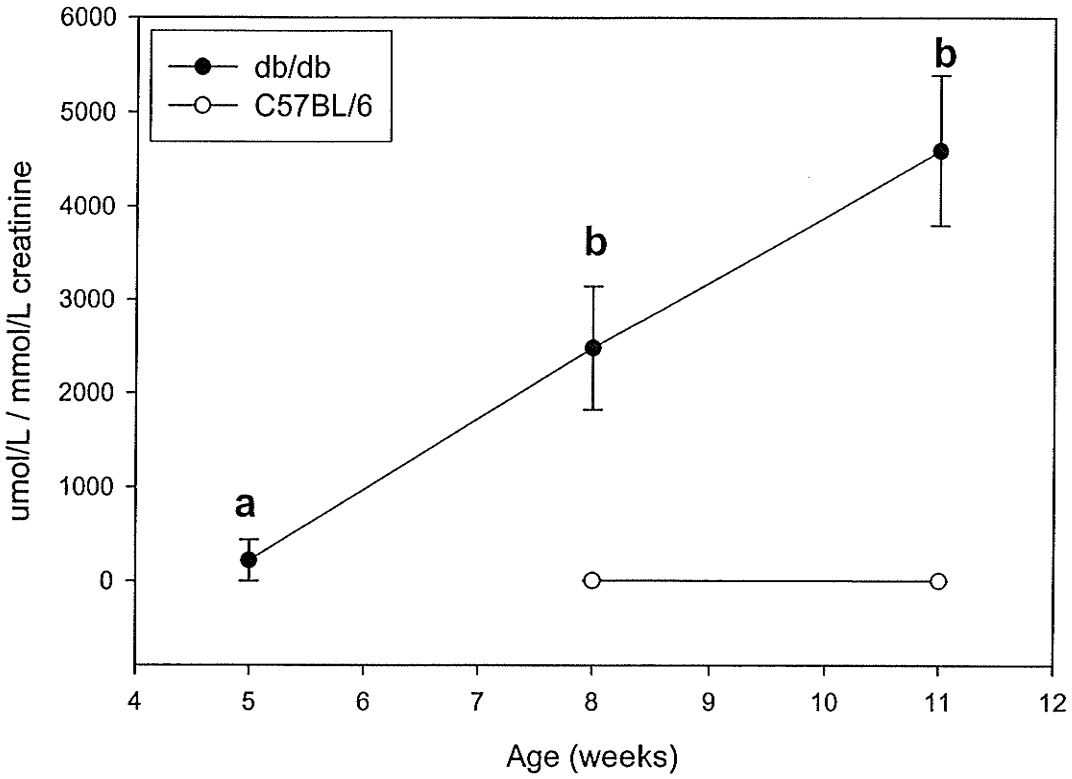


Figure 4-5. Urinary D-*chiro*-inositol (D-CI) excretion of db/db mice (closed circles) at 5, 8, and 11 weeks of age compared to C57BL/6 mice (open circles) at 8 and 11 weeks of age. For db/db mice, data points with different letters are significantly different ($p<0.05$) as determined by Duncan's multiple range test (n=4, n=5, and n=6 at 5, 8, and 11 weeks, respectively). Urinary D-CI excretion of db/db mice at 8 and 11 weeks of age was higher than in C57BL/6 mice where D-CI excretion was 5.49 ± 3.44 (n=4) at 8 weeks and 1.87 ± 0.68 (n=6) at 11 weeks, with significance at $p=0.0198$ and $p=0.0022$, respectively, as determined by the t-test.

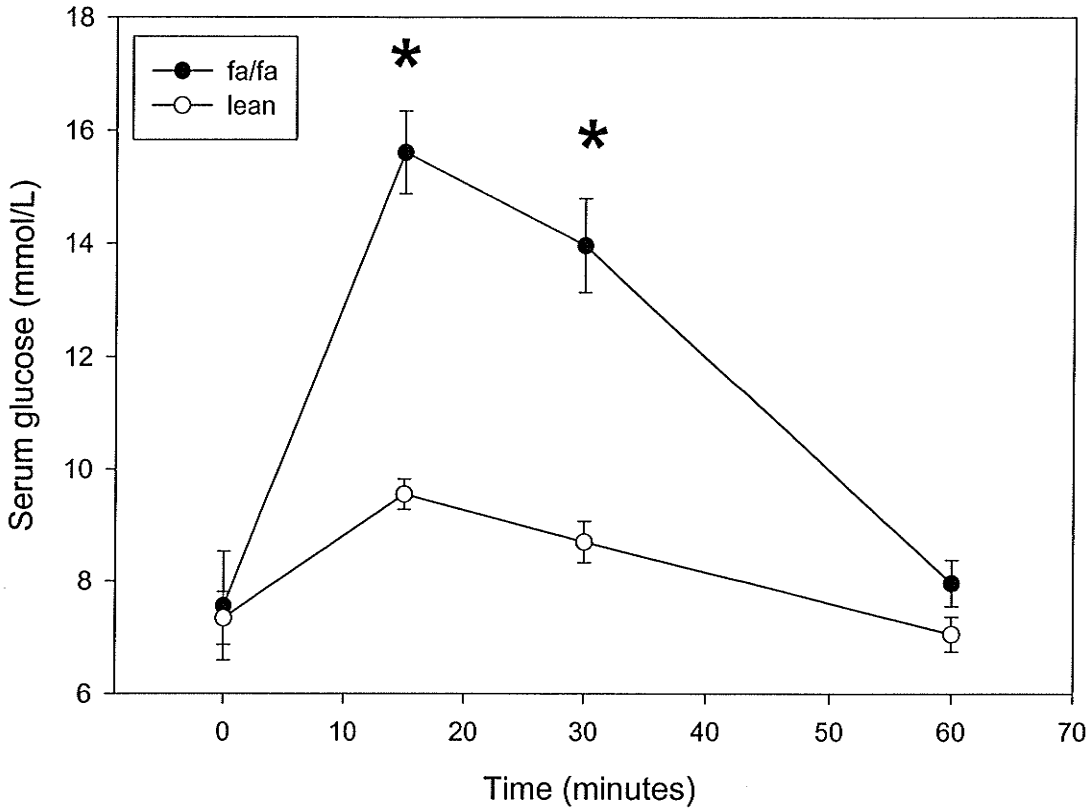


Figure 4-6. Serum glucose concentrations of fa/fa (closed circles) and lean (open circles) Zucker rats during an oral glucose tolerance test. An * indicates differences ($p<0.002$) in serum glucose concentrations, as determined by the t-test.

4.5 Discussion

The data presented show that urinary inositol excretion is substantially altered in animal models of DM-1 and DM-2, but not IGT. Table 4-4 provides a summary of the patterns of inositol excretion and diabetic indices in the different animal models. In the animal models of DM-1 and DM-2, STZ rats and db/db mice, respectively, urinary excretion of both D-CI and MI was elevated and the ratio of MI/D-CI was reduced compared to their normal counterparts. However, in the fa/fa Zucker rats, a model for IGT, urinary inositol excretion and the ratio of MI/D-CI were not altered. The fa/fa Zucker rats had hyperinsulinemia but not hyperglycemia (fasting serum glucose > 7 mmol/L) or glucosuria as was observed in the STZ rats and db/db mice. The pattern of urinary inositol excretion observed in fa/fa Zucker rats was similar to the non-diabetic animals (lean Zucker rats, normal C57BL/6 mice and Sprague-Dawley rats). In all diabetic animal models, urinary D-CI excretion was positively correlated with urine glucose. In contrast, no relationships were observed between D-CI excretion and urine glucose in normal animals.

The present study compares inositol excretion in animal models of DM-1 and DM-2. Urinary excretion of both D-CI and MI were elevated in STZ rats and db/db mice, while inositol excretion was normal in fa/fa Zucker rats. Suzuki *et al* (1991) reported elevated urinary MI excretion in diabetic Goto-Kakizaki (GK) rats, but reduced D-CI excretion compared to normal Wistar rats. GK rats are non-obese, mildly hyperglycemic (8.5 mmol/L), hyperinsulinemic, and have impaired intravenous glucose tolerance due to a poor insulin secretory response (Portha

et al, 1991). To date, this is the first study to publish data on the urinary inositol excretion in db/db mice. Although a comment in the paper of Kennington *et al* (1990) indicated that the pattern of inositol excretion in STZ and fa/fa Zucker rats is different from diabetic humans, no data were reported. Our data indicate elevated inositol excretion in diabetic animal models characterized by hyperglycemia and glucosuria, regardless of circulating insulin and body weight (hypoinsulinemic non-obese STZ rats vs. hyperinsulinemic obese db/db mice).

Urinary D-CI excretion has been reported as both reduced in human subjects with DM-2 (Kennington *et al*, 1990; Suzuki *et al*, 1994) and elevated in humans with DM-1 and DM-2 (Ostlund *et al*, 1993). Urinary MI has been consistently demonstrated as higher among individuals with DM-2 (Asplin *et al*, 1993; Shashkin *et al*, 1997; Kunjara *et al*, 1999) and DM-1 (Ostlund *et al*, 1993). In the work of Ostlund *et al* (1993), elevated urinary excretion of D-CI was strongly related to urinary glucose, plasma glucose and glycosylated hemoglobin. Findings of the present study suggest a positive relationship between D-CI excretion and glucosuria. In addition, the same authors found that urinary D-CI excretion was particularly elevated among sulfonylurea-treated DM-2 patients with poor glycemic control, and subsequent insulin treatment for 1-20 days reduced mean D-CI excretion by 63%. Similarly, D-CI excretion increases with the severity of diabetes as demonstrated in db/db mice from age 5 to 11 weeks (Figure 4-5).

In the present study, free inositols were determined using an alternate method for urine preparation than was used in previous reports (Suzuki *et al*,

1991; Kennington *et al*, 1990; Ostlund *et al*, 1993; Suzuki *et al*, 1994). The method used in the present study does not apply any purification steps in order to avoid losses of inositols, but rather it involves the derivatization of all components in urine samples using a silylation agent specific for hydroxyl groups. The pattern of MI excretion observed among diabetic animal models in the present study is similar to previous reports on humans and diabetic animal models (Suzuki *et al*, 1991; Kennington *et al*, 1990; Ostlund *et al*, 1993; Suzuki *et al*, 1994). Our results for D-CI excretion in animal models of DM-1 and DM-2 support those of Ostlund *et al* (1993) but are different from those reported by Kennington *et al* (1990), and Suzuki *et al* (1991, 1994). Further characterization of these different animal models and types of diabetes is required to understand the observed differences.

Dietary intake is a possible reason for increased urinary excretion of D-CI and MI. We analyzed both the semi-purified diet and the standard laboratory chow for presence of D-CI and MI. Neither inositol was detected in the semi-purified diet and only 0.032 mg of D-CI and 0.122 mg of MI per gram of diet were detected in the laboratory chow. The pattern of inositol excretion in STZ rats and db/db mice reported in this study is not due to differences in dietary intake. The only animal pairs not fed the same diet were the db/db mice and normal C57BL/6 mice. To the contrary, inositol excretion was higher in db/db mice fed the semi-purified diet in which no inositols were detected.

Besides dietary intake, other potential explanations for increased renal excretion of a substance include increased production, decreased tissue uptake,

or altered renal metabolism. Pak *et al* (1998) demonstrated the incorporation of radioactive labeled MI and D-CI into a wide range of tissues, with kidney being the highest. Incorporation into inositol phospholipids and inositol phosphates varied for each tissue (Pak *et al*, 1998). MI can be synthesized from glucose in several tissues including the liver and kidney (Beemster *et al*, 2002). Furthermore, the conversion of MI to D-CI has been demonstrated in various tissues including liver, muscle, fat, and kidney (Pak *et al*, 1998). In contrast, no or minimal conversion of D-CI to MI was reported. The major product of MI breakdown is glucuronic acid with kidney as the main site of the oxidation (Pak *et al*, 1998). It is possible that increased urinary D-CI and MI excretion in STZ rats and db/db is related to the metabolism of inositols in these animals.

To date, the metabolism of inositols has not been fully elucidated, however, experimental evidence suggests that inositol metabolism is altered among diabetic animal models. Pak *et al* (1998) reported a difference in the metabolism of MI and D-CI in diabetic GK rats compared to normal Wistar rats. GK rats demonstrated a defect in conversion of MI to D-CI in insulin-sensitive tissues and increased incorporation of MI and D-CI into inositol phospholipids. However, the relationship of altered inositol metabolism with urinary excretion was not examined. It is possible that altered inositol metabolism also exists among STZ rats and db/db mice, leading to elevated inositol excretion. Altered renal metabolism may also explain the increased inositol excretion in these animal models. According to Niwa *et al* (1983), urinary excretion of MI and D-CI was significantly increased in patients with chronic renal failure. Further studies

are needed to investigate inositol metabolism and possible renal perturbations in STZ rats and db/db mice.

Elevated urinary D-CI and MI excretion in STZ rats and db/db mice is also consistent with reports of impaired GPI/IPG signaling systems in diabetic animal models. Sanchez-Arias *et al* (1992) reported that hepatocytes isolated from STZ rats had 60% less GPI, blocked hydrolysis of GPI in response to insulin, and markedly reduced uptake of IPG (40%), compared to control rats. Although the exact mechanism and the role of inositols in GPI/IPG signaling is not fully understood, increased renal excretion of D-CI and MI is in keeping with this observed defect. To date, no data on the GPI/IPG signaling system has been reported for db/db mice, however, a similar pattern of impaired GPI-dependent insulin signaling was demonstrated in 14-21 week old fa/fa rats (Sanchez-Gutierrez, 1994). Hepatocyte content of GPI and IPG uptake was reduced by approximately 30% in fa/fa rats compared to lean control animals. These findings bring into question why we did not observe the same pattern of increased inositol excretion in fa/fa rats as STZ rats, if both have impaired GPI/IPG signaling systems. In the present study, the fa/fa rats were only 8 weeks old and may not have a sufficiently impaired GPI/IPG signaling system. However, we did observe altered inositol excretion in STZ rats and db/db mice at 8 weeks of age. Another possible explanation is that the pattern of impaired GPI/IPG signaling in hepatocytes of fa/fa rats reported by Sanchez-Gutierrez *et al* (1994) was less dramatic than the STZ rats (Sanchez-Arias *et al*, 1992) and is concomitant with normoglycemia of fa/fa rats. Furthermore, results from the

present study indicate that urinary D-CI and MI excretion increases with the severity of diabetes, as observed in the db/db mice. Nonetheless, if an impaired GPI/IPG signaling system is responsible for increased inositol excretion, then the impaired signaling system observed in both STZ rats (insulin-deficient) and fa/fa rats (insulin-resistant) can explain why urinary inositol excretion was greater in both STZ rats and insulin resistant db/db mice.

The data presented here suggest that urinary D-CI and MI excretion is elevated in animal models of DM-1 and DM-2, both characterized by hyperglycemia and glucosuria. It remains to be elucidated whether altered inositol metabolism of diabetic animal models relates to the pathogenesis of diabetes and the potential mechanisms involved. It is still unclear if the same pattern of urinary inositol excretion exists among humans with diabetes.

5. Urinary *myo*-inositol excretion is elevated in individuals with type 1 diabetes mellitus and elevated *myo*- and D-*chiro*-inositol excretion is related to hyperglycemia and glucosuria.

Kawa J, Przybylski R, Taylor C.

5.1 Abstract

The bioactivity of insulin mediators, containing D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) is altered in diabetes mellitus (DM). We previously reported that urinary excretion of D-CI and MI was elevated in animal models of type 1 and type 2 DM (DM-1 and DM-2, respectively) that had hyperglycemia and glucosuria. The purpose of the present study was to determine whether the same pattern of inositol excretion exists in humans with diabetes. We analyzed the 24-hour urinary excretion of inositols in individuals with DM-1 and DM-2 compared to individuals without DM, matched for gender, age and BMI. Individuals with DM-1 had higher urinary D-CI and MI excretion as compared to their matched controls (308 ± 133 μM/24 hours and 757 ± 132 μM/24 hours versus 19.1 ± 7.9 μM/24 hours and 102 ± 20 μM/24 hours). In contrast, urinary inositol excretion in individuals with DM-2 was not different from their matched control group, or the DM-1 control group. The DM-2 group in the present study, however, was no different from either control group for glycosylated hemoglobin ($HbA1_c$) or urinary glucose, suggesting that they did not have hyperglycemia or glucosuria. This is in keeping with the fact that DM-2 participants were managed by diet only, which suggests their diabetes was not severe. In this study, significant relationships between urinary inositol excretion versus $HbA1_c$ and urinary glucose were also observed. Thus, our results indicate that elevated urinary D-CI and MI is present in individuals with DM, and is concomitant with hyperglycemia and glucosuria. This is the same pattern we previously observed in animal models of DM.

5.2 Introduction

In a previous study, we reported that urinary excretion of D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) was elevated in animal models of type 1 and type 2 diabetes mellitus (DM-1 and DM-2, respectively) (Kawa *et al*, 2003; Chapter 4). Both D-CI and MI have been identified as components of inositol phosphoglycan (IPG) molecules that have demonstrated insulin mimetic activities. Over the last two decades, the potential role of these inositol isomers and their IPGs in DM has been the subject of numerous investigations and has been reviewed elsewhere (Field, 1997; Jones and Varela-Nieto, 1999). Impaired IPG release has been reported in animal models of DM-1 and DM-2 (Suzuki *et al*, 1991; Sanchez-Arias *et al*, 1992; Sanchez-Gutierrez *et al*, 1994). Altered bioactivity of IPGs has also been documented among individuals with DM-1 and DM-2 (Asplin *et al*, 1993; Shashkin *et al*, 1997, Kunjara *et al*, 1999). Impaired inositol metabolism in the diabetic Goto-Kakizaki rat has also been reported (Pak *et al*, 1998).

In accordance with altered IPG activity and inositol metabolism in DM, urinary excretion of D-CI and MI also appears to be altered in DM. Elevated MI excretion has been consistently reported among individuals with DM-1, DM-2 and impaired glucose tolerance (IGT) (Kennington *et al,* 1990; Ostlund *et al*, 1993; Suzuki *et al*, 1994) compared to individuals with normal glucose tolerance. In contrast, reports differ on the pattern of urinary D-CI excretion in DM. Reduced urinary D-CI has been reported in humans with DM-2 (Kennington *et al,* 1990; Suzuki *et al*, 1994) and IGT (Suzuki *et al*, 1994) while Ostlund *et al* (1993) found

that both type 1 and type 2 diabetic patients had elevated urinary D-CI excretion compared with normal subjects. Kennington *et al* (1990) stated that analyses of urine from individuals with DM-1 revealed a wide variation in D-CI excretion but no data were given. Results from our laboratory suggest that urinary excretion of both D-CI and MI were elevated in diabetic db/db mice and streptozotocin (STZ) rats, models of DM-2 and DM-1, respectively. In contrast, there was no difference in D-CI or MI excretion in fa/fa Zucker rats, a model of IGT, compared to their lean counterparts (Kawa *et al*, 2003; Chapter 4).

The purpose of the present investigation was to determine whether the pattern of urinary inositol excretion we previously observed in animal models of DM is the same in humans with DM-1 and DM-2. Previously published studies have used extensive sample purification procedures prior to urine inositol analysis whereas our method does not employ any sample purification steps to avoid losses and possible changes due to isomerization. In addition, we determined urinary excretion of glucose and glucuronic acid in the present study. In mammals, MI can be endogenously synthesized from glucose and the breakdown of MI results in glucuronic acid, with the kidney being the main site of oxidation (Beemster *et al*, 2002; Pak *et al*, 1992). Thus, the objective of this study was to determine the 24-hour urinary excretion of MI, D-CI, glucose and glucuronic acid in individuals with DM-1 and DM-2 compared to individuals without DM, matched for gender, age, and body mass index. Clarification of the pattern of urinary inositol excretion can contribute to the understanding of the altered metabolism and bioactivity of these compounds that exists in DM. This

can also provide further knowledge on the role of these compounds in the pathogenesis of DM which may lead to novel treatments for the disease.

5.3 Materials and Methods

Standards and chemical reagents

The *myo*-inositol standard, glucose, glucuronic acid, phenyl-α-D-glucoside (internal standard), trimethylsilylimidazole, and pyridine were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent alcohol was purchased from Fisher Scientific (Ontario, Canada). D-*chiro*-inositol standard was purchased from Industrial Research Limited (Lower Hutt, New Zealand).

Participants

The pattern of urinary inositol excretion was determined in 24-hour urine samples obtained from individuals with and without DM-1 and DM-2. Volunteers were recruited from the University of Manitoba (Winnipeg, Manitoba, Canada) and surrounding community through advertising. Participants with self-declared diabetes were matched to individuals without diabetes according to gender, age, and body mass index (BMI). Individuals with DM-1 were free of all other medical conditions besides diabetes and their matched control counterparts did not have any medical conditions. Participants with DM-2 were managed by diet and were not taking any oral antidiabetic agents or insulin. Some participants within the group of DM-2 subjects and their matched controls had hypertension and/or hypercholesterolemia, but were free of other medical conditions. All study procedures were approved by the Biomedical Research Ethics Board at the University of Manitoba. Subjects volunteered to participate in the study and the appropriate consent was obtained.

Urine collection

Twenty-four hour urine samples were collected into 4 L disposable specimen collection containers (Fisher Scientific) without cooling. Ostlund *et al* (1993) reported that inositols are stable during urine collection. This was confirmed in our laboratory (data not shown). The volume of each 24-hour urine sample was calculated using weight and aliquots were removed immediately following the 24-hour collection period and stored at -80°C until analysis.

Blood collection and analysis of glycosylated hemoglobin

Blood samples were obtained from participants immediately following the 24-hour urine collection. Blood collection was performed by trained medical personnel at the St. Boniface General Hospital (Winnipeg, Manitoba, Canada). Determination of glycosylated hemoglobin ($HbA1_c$) in blood samples was performed by the biochemistry laboratory at the St. Boniface General Hospital.

Preparation of urine for analysis

The D-CI, MI, glucose and glucuronic acid content was determined in 24-hour urine specimens collected from study participants according to the method described by Kawa *et al* (2003) (Chapter 4, Section 4.3). Briefly, 0.5 mL of ethanol was added to an equal volume of urine. Samples were vortexed and evaporated to dryness under nitrogen at 40°C. Dried samples were sonicated for 5 minutes with 1 mL of trimethylsilylmidazole (TMS):pyridine (1:1, v/v) which contained 200 μg phenyl-α-D-glucoside as an internal standard, and derivatized for 1 hour at 80°C.

Urine analysis by gas chromatography

Two μL of derivatized samples or standards were injected into a Shimadzu gas chromatograph model GC-17A (Columbia, Maryland) equipped with a flame ionization detector and split injector. TMS-derivatives were separated on a RTX-5MS capillary column (25 m length, 0.25 mm ID, and 0.25 μm film thickness; Restek, Bellefonte, PA). Column temperature was programmed from 150°C to 200°C at the rate of 3°C/min, then to 325°C at the rate of 7°C/min. Initial and final temperatures were held for 5 and 20 minutes, respectively. The injector and detector temperatures were held at 270°C and 350°C, respectively. The carrier gas was hydrogen at 1.5 ml/min while the split ratio used was 1:40. Inositols, glucose and glucuronic acid were quantified using phenyl-α-D-glucoside as the internal standard. Standard curves for D-CI and MI were linear from 1 to 100 μg with r^2 values of 0.999 and an average error of 3.18% and 3.68%, respectively. The standard curve for glucose was linear from 1 to 1000 μg with an r^2 value of 0.999 and an average error of 3.47%. The standard curve for glucuronic acid was linear from 1 to 500 μg with an r^2 value of 0.998 and an average error of 3.38%. Urine samples were analyzed in duplicate.

Statistical analysis

Statistical significance between the participant groups was determined by an ANOVA and by Duncan's multiple range test for means testing (SAS v.9.1, SAS Institute Inc., Cary, NC). Correlations were analyzed using Pearson's correlation coefficient. Differences were accepted as significant at $p<0.05$. Data are expressed as the mean ± SE.

5.4 Results

In the present study, we determined the 24-hour urinary excretion of D-CI and MI in individuals with DM-1 and DM-2 compared to individuals without DM matched for gender, age, and BMI. For participants with DM-1 compared to their matched control group, there were no differences in age, height, weight and BMI (Table 5-1) whereas significant differences were found between these two groups for the diabetic indices ($HbA1_c$ and urinary glucose), as expected (Table 5-2). The DM-2 group was also not different from their matched control group for age, height, weight and BMI (Table 5-1). Interestingly, there was no difference between the DM-2 group and their matched non-diabetic controls for $HbA1_c$ and urinary glucose (Table 5-2). Compared to the group with DM-1, the DM-2 group had significantly lower values for $HbA1_c$ and urinary glucose, and was not different from the DM-1 control group for these indices (Table 5-2). This suggests that the DM-2 control group in the present study had relatively normal values for $HbA1_c$ and urinary glucose.

Individuals with DM-1 had higher urinary excretion of both MI and D-CI as compared to their matched control group, the DM-2 group, and the DM-2 matched controls (Table 5-2 and Figure 5-1). The urinary excretion of MI was 757 ± 132 μmol/24 hours in individuals with DM-1 versus 102 ± 20 μmol/24 hours in the DM-1 control participants (Figure 5-1A). In the DM-1 group, urinary D-CI excretion was 308 ± 133 μmol/24 hours versus 19.1 ± 7.9 μmol/24 hours in the DM-1 control group (Figure 5-1B). As shown in Table 5-2, the same pattern was observed when MI and D-CI were expressed as urinary concentrations (μM)

rather than μmol amounts per 24 hours. In contrast to the pattern of elevated inositol excretion observed in DM-1 participants, mean urinary MI and D-CI levels were not different between individuals with and without DM-2 (Table 5-2, Figure 5-1A and B). Furthermore, urinary inositol excretion in DM-2 participants was not different from the DM-1 control group (Table 5-2, Figure 5-1A and B).

In addition to elevated inositol excretion, urinary glucose and glucuronic acid were also significantly higher in the group with DM-1 compared to all other groups (Table 5-2, Figure 5-1C and D). There was, however, no difference in the ratio of MI/D-CI between all four groups. A t-test was also performed on the data to compare each DM group with their matched controls. Similar to the ANOVA, the t-test revealed that the DM-1 group was significantly different from their matched controls for $HBA1_c$, urinary glucose, D-CI, MI whereas there were no differences between the DM-2 group and their matched controls for these measurements. Therefore, we performed an ANOVA to compare all 4 groups, thus allowing the DM-2 group and their controls to be compared to the groups with and without DM-1, which had a normal BMI.

We also examined the relationships between diabetic indices (urinary glucose and $HbA1_c$) and urinary inositol and glucuronic acid excretion for the entire study population. Both urinary MI and D-CI excretion were positively correlated with the diabetic indices (Table 5-3; Figure 5-2). Significant relationships were also observed between urinary glucuronic acid versus urinary glucose and $HbA1_c$ (Table 5-3).

Table 5-1. Characteristics of individuals with and without DM-1 and DM-2.

	DM-1	DM-1 Control	DM-2	DM-2 Control
Female/Male	5/1	5/1	4/5	4/5
Age (years)	22.8 ± 2.0^{b}	25.0 ± 1.5^{b}	49.6 ± 3.5^{a}	47.0 ± 3.3^{a}
Height (cm)	170 ± 3^{a}	169 ± 4^{a}	175 ± 5^{a}	178 ± 3^{a}
Body weight (kg)	75.6 ± 8.5^{ab}	71.8 ± 7.0^{b}	96.1 ± 7.2^{a}	95.5 ± 6.0^{a}
BMI (kg/cm^2)	25.9 ± 1.9^{ab}	25.0 ± 1.4^{b}	31.0 ± 1.4^{a}	29.9 ± 1.8^{ab}

Values are means ± SE. Means with different superscript letters in each row are significantly different as determined by Duncan's multiple range test ($p<0.05$).

Table 5-2. Urine and serum measurements in individuals with and without DM-1 and DM-2.

	DM-1	DM-1 Control	DM-2	DM-2 Control
$HbA1_c$ (%)	8.42 ± 0.55^a	5.42 ± 0.21^b	6.23 ± 0.22^b	6.16 ± 0.13^b
Urinary D-CI (μM)	163 ± 74^a	12.4 ± 6.9^b	6.2 ± 7.5^b	12.5 ± 3.5^b
Urinary MI (μM)	384 ± 71^a	60.3 ± 16.4^b	124 ± 47^b	66.7 ± 10.1^b
Urinary glucose (mM)	28.0 ± 9.1^a	0.06 ± 0.02^b	0.06 ± 0.03^b	0.01 ± 0.00^b
Urinary glucuronic acid (μM)	1070 ± 323^a	93.7 ± 38.5^b	141 ± 53^b	87.0 ± 20.0^b
Urinary MI/D-CI	4.19 ± 1.63^a	7.42 ± 1.78^a	9.66 ± 2.11^a	8.05 ± 2.00^a

Values are means ± SE. Means with different superscript letters in each row are significantly different as determined by Duncan's multiple range test ($p<0.05$).

Table 5-3. Relationships between diabetic indices ($HbA1_c$ and urinary glucose) versus urinary inositol and glucuronic acid excretion.

	$HbA1_c$	Urine glucose (mM)	Urine glucose (mM/24 hours)
Urinary D-CI (μM)	0.72	0.94	0.93
Urinary D-CI (μM/24 hours)	0.72	0.93	0.93
Urinary MI (μM)	0.81	0.71	0.72
Urinary MI (μM/24 hours)	0.78	0.67	0.69
Urinary Glucuronic acid (μM)	0.78	0.98	0.97
Urinary Glucuronic acid (μM/24 hours)	0.78	0.98	0.98

Correlations are expressed as Pearson's correlation coefficient (*r*) and were significant ($p<0.0001$) for n=30.

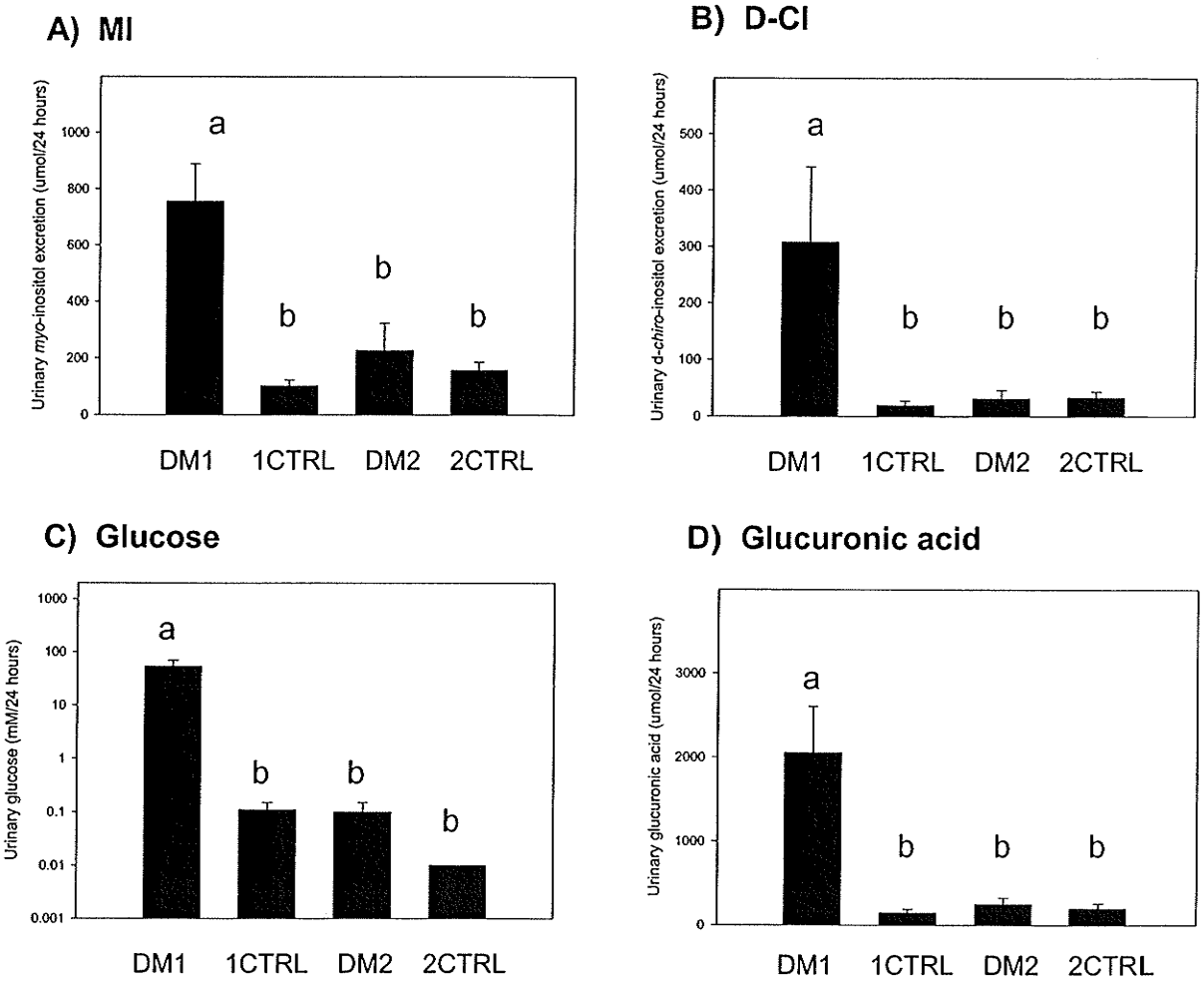
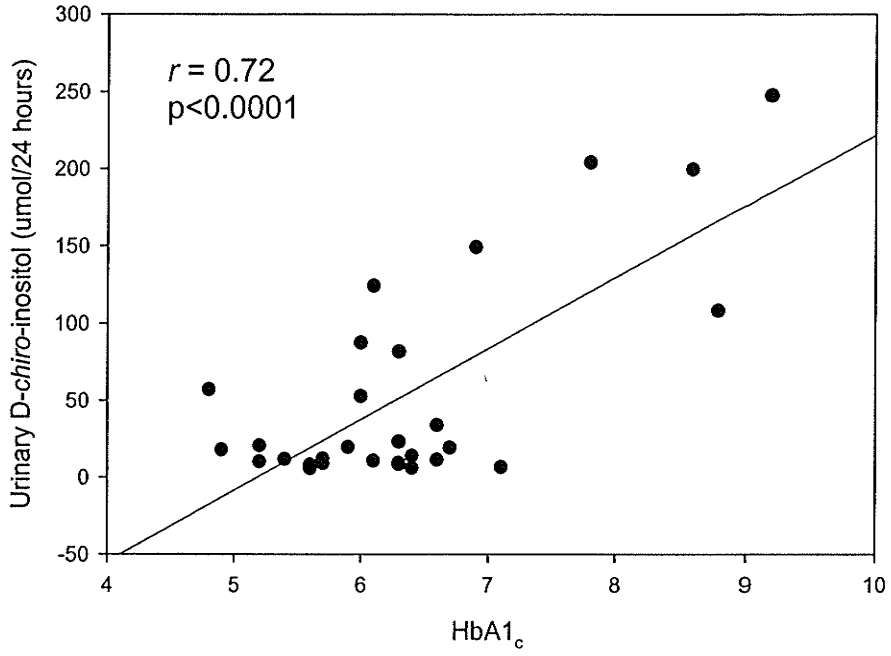


Figure 5-1. Urinary excretion of A) *myo*-inositol (MI), B) D-*chiro*-inositol (D-CI), C) glucose and D) glucuronic acid in individuals with DM-1 and DM-2 versus individuals without DM matched for gender, age and BMI (1CTRL and 2CTRL for DM-1 and DM-2, respectively). Urinary MI, D-CI, and glucuronic acid are expressed as μM/24 hours and urinary glucose is expressed as mM/24 hours. Different letters indicate significant differences between groups as determined by Duncan's multiple range test ($p<0.05$).

A)



B)

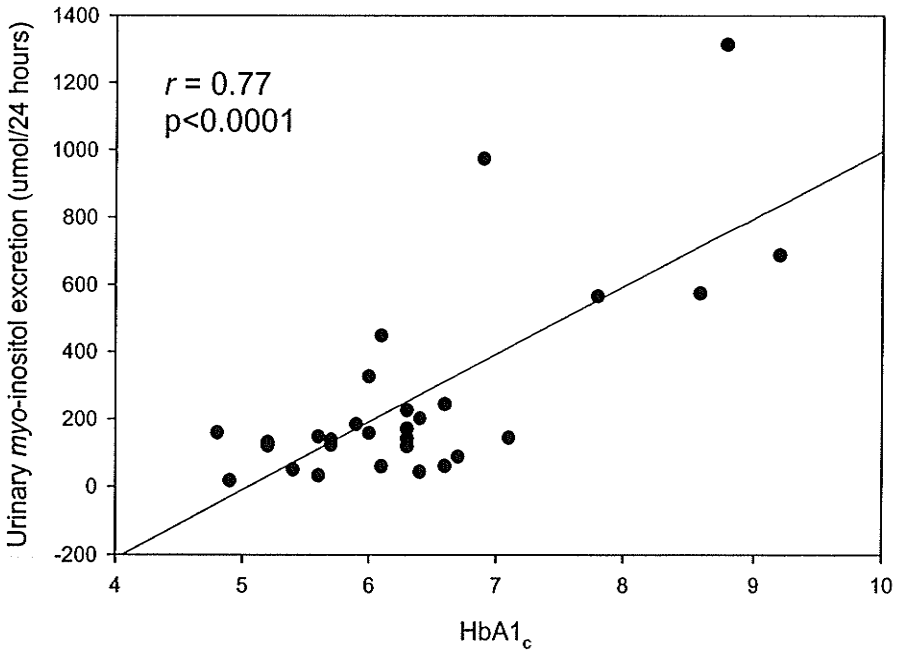


Figure 5-2. Scatter plots of urinary A) D-*chiro*-inositol versus $HbA1_c$ and B) *myo*-inositol versus $HbA1_c$ in individuals with and without DM-1 and DM-2. Data points represent the values obtained from individual participants (n=30).

5.5 Discussion

The main finding of this study was that the urinary excretion of D-CI and MI was significantly elevated in individuals with DM-1. Participants with DM-1 also had significantly higher $HbA1_c$, urinary glucose and glucuronic acid excretion compared to their control counterparts. In contrast, urinary D-CI and MI were not elevated in the group with DM-2, however, there were also no differences between the DM-2 group versus their control counterparts for $HbA1_c$ or urinary glucose. Significant relationships between urinary D-CI, MI, and glucuronic acid excretion versus diabetic indices ($HbA1_c$ and urinary glucose) were also observed among participants in this study. Thus, elevated inositol excretion appears to be related to hyperglycemia and glucosuria. This is the same pattern we previously observed in diabetic animal models (Kawa *et al*, 2003; Chapter 4).

Our findings are also similar to those of Ostlund *et al* (1993) who reported that urinary D-CI and MI excretion was elevated among individuals with DM-1 compared to those without. In that study, the mean urinary MI excretion for DM-1 subjects was 825 μmol/24 hrs with the data ranging from 159-1554 μmol/24 hrs. The mean urinary MI excretion for individuals with DM-1 in the present study was 757 μmol/24 hrs, with the data ranging from 448-1310 μmol/24 hrs. According to Ostlund *et al* (1993), the mean urinary excretion of D-CI was 74.0 μmol/24 hrs, with the data ranging from 1.5-320 μmol/24 hrs. In the present study, the mean urinary D-CI excretion for the DM-1 group was 308 μmol/24 hrs (data ranging from 108-965 μmol/24 hrs) which is higher than reported by Ostlund *et al* (1993).

However, in both the present study and that of Ostlund *et al* (1993), mean urinary D-CI excretion was elevated in DM-1 compared to the control group. To date, these are the only reports on urinary inositol excretion in DM-1. It is possible that the higher D-CI excretion observed in our study compared to that of Ostlund *et al* (1993) is because we measured *chiro*-inositol (the sum of D- and L- isomers) whereas Ostlund *et al* (1993) measured D-*chiro*-inositol and L-*chiro*-inositol separately. However, according to Ostlund *et al* (1993), the majority of *chiro*-inositol in urine is from the D-isomer thus the difference between our reports is not likely due to the L-isomer.

In the present study, urinary inositol excretion in participants with DM-2 was not different from their matched control group, nor was it different from the DM-1 control group. This is unlike previous reports where urinary MI excretion has been elevated in individuals with DM-2, and D-CI excretion has been both elevated and reduced in DM-2 compared to non-diabetic groups (Kennington *et al*, 1990; Suzuki *et al*, 1994; Ostlund *et al*, 1993). In our study, we also did not find any differences in $HbA1_c$ or urinary glucose between the DM-2 group versus their matched controls and the DM-1 controls. Individuals with DM-2 in this study were had self-declared diabetes and were not diagnosed with diabetes as part of the study procedures. Furthermore, these participants were managed by diet and were not taking any oral medications or insulin. This suggests that our DM-2 group either had well-controlled diabetes, is within the early stages of the disease, or were not in fact diabetic, which may have contributed to the lack of differences observed in the present study for DM-2 participants versus controls.

Unlike the DM-2 participants in this study, DM-2 subjects in previous studies were taking oral medications and/or insulin therapy and had elevated $HbA1_c$ values compared to the control groups. $HbA1_c$ values can indicate management of DM by providing a measure of blood glucose control over the previous 2-3 months. $HbA1_c$ values between 4-6% are considered ideal for a non-diabetic individual whereas the optimal $HbA1_c$ value of less than 7% indicates good control of diabetes. Suboptimal glucose control is considered when $HbA1_c$ values are 7-9%, and a value greater than 9% is indicative of poorly controlled diabetes (Meltzer *et al*, 1998). Ostlund *et al* (1993) reported that urinary D-CI excretion was particularly elevated among sulphonylureas-treated DM-2 patients with poor glycemic control. In the present study, we excluded one DM-2 participant from our analysis that had a high $HbA1_c$ value (13.4%) relative to other participants in the DM-2 group, however, this individual had high urinary D-CI and MI excretion (39,000 and 9343 μmol/24 hours, respectively).

The relationship between urinary inositol excretion and diabetic indices in the present study is in keeping with our observations on the urinary inositol in diabetic animal models (Kawa *et al*, 2003; Chapter 4). We previously reported that urinary D-CI and MI excretion was elevated in the diabetic db/db mouse and streptozotocin (STZ) rat, but not the fa/fa Zucker rat. STZ rats are a model of DM-1, characterized by hyperglycemia, glucosuria, and hypoinsulinemia. The db/db mouse is a model of DM-2, characterized by obesity, hyperglycemia, glucosuria, and hyperinsulinemia. The fa/fa Zucker rats are obese, have

impaired glucose tolerance and are a model of the early stages of DM-2. These animals are hyperinsulinemic but do not have the extreme hyperglycemia or glucosuria observed in the db/db mice and STZ rats. Thus, in the previous study we concluded that the elevated urinary D-CI and MI excretion was concomitant with hyperglycemia and glucosuria. Similar results were observed in humans in the present study.

In an attempt to further understand the altered metabolism of inositols in diabetes, we also determined urinary excretion of glucuronic acid, a product of MI catabolism, and found that the DM-1 group also had elevated urinary glucuronic acid compared to the other groups. Chronic hyperglycemia results in glucosuria after the renal threshold for glucose reabsorption is reached. In the present study, only the DM-1 group had significant hyperglycemia and glucosuria. Glucose can be converted to MI, and MI can be converted to either D-CI or glucuronic acid (Beemster *et al*, 2002; Pak *et al*, 1992). Thus, the elevated inositol and glucuronic acid excretion observed among individuals with DM-1 may be related to increased production of these metabolic products in the kidney resulting from hyperglycemia and glucosuria. This would also explain why we did not observed elevated inositol or glucuronic acid in the other groups in this study, as they did not have glucosuria. Another possible reason for elevated inositol excretion may be related to a defect in MI and D-CI incorporation into IPG precursors that exists in diabetes.

Results from this study indicate that urinary MI and D-CI are elevated in DM-1 and that urinary inositol excretion is positively related to hyperglycemia and

glucosuria. Future studies can determine the contribution of this urinary inositol pattern to the pathogenesis of the disease and can clarify the pattern of urinary inositol excretion in DM-2 versus IGT, or the early stages of the disease.

6. Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats

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Kawa J, Taylor C, Przybylski R. Buckwheat concentrate reduces serum glucose in streptozotocin-diabetic rats. Journal of Agricultural and Food Chemistry. 2003. 51: 7287-7291.

6.1 Abstract

The antihyperglycemic effects of chemically synthesized D-*chiro*-inositol (D-CI), a component of an insulin mediator, have been demonstrated in rats. Buckwheat contains relatively high levels of D-CI, thus it has been proposed as a source of D-CI for reducing serum glucose concentrations in diabetics. In the present study, we evaluate the effects of a buckwheat concentrate (BWC), containing D-CI, on hyperglycemia and glucose tolerance in streptozotocin (STZ) rats. In fed STZ rats, both doses of the BWC (containing 10 and 20 mg D-CI/kg body weight) were effective for lowering serum glucose concentrations by 12-19% at 90 and 120 minutes after administration. Findings from this study demonstrate that a BWC is an effective source of D-CI for lowering serum glucose concentrations in rats and therefore may be useful in the treatment of diabetes.

6.2 Introduction

Of nine inositol isomers, *myo*-inositol (MI) is the most commonly occurring isomer in nature, whereas D-*chiro*-inositol (D-CI) is relatively rare. Buckwheat seeds contain relatively high amounts of free D-CI and galactosyl derivatives of D-CI known as fagopyritols (Horbowicz and Obendorf, 1994). Horbowicz and Obendorf (1994) detected free D-CI in lupine, pigeon pea, soybean, chickpea, mungbean, and buckwheat. Among all the seeds analyzed, only mungbean seeds contained higher levels of free D-CI than buckwheat. According to Horbowicz *et al* (1998), one of the fagopyritols has been identified in soybean, lupine, lentil, and chickpea seeds whereas buckwheat contains five different fagopyritols.

Besides its existence in plant sources, D-CI and MI have been identified as components of two different inositolphosphoglycan (IPG) molecules in mammalian systems (Larner *et al*, 1988; 1989). The role of IPG molecules as putative insulin secondary messengers has been demonstrated in numerous studies (Varela-Nieto *et al*, 1996; Field, 1997; Jones and Varela-Nieto, 1998; 1999). IPGs are released from glycosylphosphatidylinositols (GPIs) in cell membranes in response to insulin. Following GPI hydrolysis by phospholipases, IPGs are incorporated into the cell where they can affect enzymes implicated in insulin action. The insulin-mimetic effects of IPGs isolated from various mammalian tissues and their analogues have been widely documented (Varela-Nieto, 1999).

The use of buckwheat in the management of diabetes mellitus has been previously reported. Consumption of buckwheat as flour or biscuits made from buckwheat flour has been demonstrated to have hypoglycemic effects in patients with diabetes (Lu *et al,* 1992; Wang *et al,* 1992), however, the active components were not identified.

The effectiveness of acute administration of chemically synthesized D-CI on lowering plasma glucose has been evaluated in rats. A single dose of intragastric D-CI (2-15 mg/kg) administered to normal rats 2 hours before intraperitoneal glucose produced a 30-50% decrease in plasma glucose concentrations (Ortmeyer *et al*, 1993). A single dose of intragastric D-CI (10 mg/kg) administered to fed streptozotocin (STZ)-treated rats produced a 30-40% decrease in plasma glucose concentrations (Ortmeyer *et al*, 1993). The acute effects of D-CI on plasma glucose were also demonstrated in STZ rats when a single dose of 15 mg/kg attenuated elevated plasma glucose concentrations by 21% in 120 minutes (Fonteles *et al*, 2000). Ortmeyer *et al* (1993) administered acute doses of 1-30 mg D-CI/kg body weight in STZ rats and found maximal effects for plasma glucose lowering at 10 mg D-CI/kg body weight. Fonteles *et al* (2000) reported significant plasma glucose lowering in STZ rats with 15 mg D-CI/kg body weight but not a 5 mg D-CI/kg dose. These studies used chemically synthesized D-CI. However, buckwheat contains sufficient amounts of D-CI to be evaluated as a natural source of D-CI for reducing serum glucose concentrations in diabetes.

The purpose of the present study was to evaluate the effect of an acute dose of a buckwheat concentrate (BWC) on elevated serum glucose and glucose tolerance in rats. To determine the efficacy of buckwheat as a natural source of D-CI, we administered the BWC to normal and fed STZ rats under conditions similar to those used in studies mentioned above. We also administered the BWC to fasted rats, prior to an oral glucose tolerance test (OGTT). Performing an OGTT is beneficial because it provides a functional measurement of whole body glucose tolerance. We hypothesized that a BWC equivalent to 10 or 20 mg D-CI/kg body weight would improve glucose tolerance in normal and STZ rats, and decrease hyperglycemia in fed STZ rats. Thus, the specific objectives of this study were 1) to determine the effectiveness of a BWC as a natural source of D-CI on glucose tolerance in normal rats and on hyperglycemia in fed STZ rats, and 2) to determine the effect of an acute dose of a BWC given to fasted rats 1 hour before an OGTT on serum glucose concentrations.

6.3 Materials and Methods

Standards and chemical reagents

The *myo*-inositol standard, phenyl-α-D-glucoside (internal standard), trimethylsilylimidazole, pyridine, and streptozotocin (STZ) were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent alcohol was purchased from Fisher Scientific (Ontario, Canada). D-*chiro*-inositol standard was a gift from Dr. S.G. Angyal (University of New South Wales, Australia).

Buckwheat concentrate

Buckwheat variety Koto (*Fagopyrum esculentum,* Moench) was provided by Kade Research Ltd. (Morden, Manitoba, Canada). Concentrates were produced from buckwheat according to a modified method of Horbowicz and Obendorf (1994). Briefly, dehulled buckwheat groats were fractionated by milling on a Buhler mill (MLU-202). Further details of the milling procedure are described in Appendix A. The bran and shorts fractions were combined, ground, and thoroughly homogenized in ethanol:water (1:1, v/v) for 10 minutes. Five volumes of extraction solvent were used for every 1 volume of ground seed fractions. The homogenate was vacuum filtered and the remaining residue re-extracted with the same volume of solvent. The combined filtrates were evaporated using a rotary evaporator (Yamato RE200, Orangeburg, NY) under vacuum until a 40-fold reduction in solvent volume was achieved. This form was used for acute administration to rats in the present study.

Analysis of inositols and soluble carbohydrates in buckwheat concentrate

Aliquots (100 μL) of the concentrate solution were transferred to silylation vials (Pierce) and evaporated to dryness under nitrogen at 40°C. The dry residues were derivatized with 1.6 mL of silylation reagent (trimethylsilylimidazole:pyridine, 1:1, v/v, containing 200 μg phenyl-α-D-glucoside) at 75-80°C for 1 hour (Horbowicz and Obendorf, 1994).

Two μL of derivatized carbohydrates were injected into a Shimadzu gas chromatograph GC-17A (Columbia, Maryland) equipped with a flame ionization detector and split injector. Carbohydrates were separated on a RTX-5MS capillary column (25 m length, 0.25 mm ID, and 0.25 μm film thickness; Restek, Bellefonte, PA). Column temperature was programmed from 150° to 200°C at the rate of 3°C/min, then to 325°C at the rate of 7°C/min. Initial and final temperatures were held for 5 and 20 minutes, respectively. The injector and detector temperatures were held at 270°C and 350°C, respectively. The carrier gas was hydrogen at a flow rate of 1.5 mL/min while the split ratio used was 1:40. Soluble carbohydrates including inositols were quantified using phenyl-α-D-glucoside as the internal standard.

Protein and mineral composition of buckwheat concentrates

Concentrates were analyzed for content of protein using AOAC method 955.04 (AOAC, 1997) and minerals by atomic absorption spectrometry.

Animals and treatment groups

Fifty-two male Sprague-Dawley rats (Central Animal Holding, Winnipeg, Manitoba, Canada) weighing 150-180 grams were acclimatized for a period of 7

days. Throughout the acclimatization and subsequent study period, rats were maintained in a controlled environment of 21-23°C, 55% humidity, and a 14-hour light, 10 hour dark cycle. Rats were housed in groups of two in plastic hanging cages, fed standard laboratory chow (Prolab RMH 3000, Purina Mills, Richmond, Indiana) *ad libitum* and fresh water was available in polypropylene bottles with stainless steel sipper tubes. Rats were familiarized with subsequent testing procedures during this adaptation period. A protocol for animal care procedures was approved by the University of Manitoba Protocol Management and Review Committee.

Treatments were a low dose BWC, high dose BWC, low dose placebo, or high dose placebo. The low dose and high dose buckwheat groups received a single dose of BWC containing 10 or 20 mg D-CI/kg body weight, respectively. The low dose and high dose placebo groups received a sucrose:deionized water solution (3% and 6% sucrose, respectively) that was administered at a dose level equivalent to the amount of sucrose provided in the BWC. For rats assigned the lower dose (10 mg D-CI/kg body weight), the BWC was diluted 2-fold so that equivalent volumes of the concentrate were administered to rats in both treatment groups.

Normal rats given a glucose load

Following the acclimatization period, 12 male Sprague-Dawley rats were randomly assigned to either the low dose BWC group or the low dose placebo group. Following treatment administration, rats were fasted for 2 hours and given an intraperitoneal glucose tolerance test (IPGTT). For the IPGTT, blood was

collected from the saphenous vein for the 0 minute time point and immediately following, a 70% glucose solution was injected intraperitoneally (4 g glucose/kg body weight). Blood was collected from the saphenous vein at 30, 60, 90, and 120 minutes from the time of the initial glucose administration. The total volume of blood samples collected during the IPGTT was less than 10% of the blood volume of rats. Blood samples were held on ice until centrifuged to obtain serum. Serum samples were stored at –20°C until analysis. The protocol for the test procedure in normal rats is shown in Figure 6-1A.

Streptozotocin rats

Following the acclimatization period, 40 rats received intraperitoneal injections of 60 mg STZ/kg body weight/day on day 1 and 2 of the experiment. STZ was freshly dissolved in 0.9% NaCl, pH 5.5 at a concentration of 15 mg/mL. Three days after the second injection, a blood sample to measure blood glucose levels was taken via the saphenous vein. Diabetes was defined when a blood glucose concentration of 13 mmol/L or greater was achieved. Rats with a positive response to STZ administration were randomly assigned to either the low dose BWC, high dose BWC, low dose placebo, or high dose placebo group. Rats were individually housed for the remainder of the experiment. Two different test procedures were performed on STZ rats. The protocols for test procedures in STZ rats are shown in Figure 6-1B and 6-1C.

Effect of buckwheat concentrate on serum glucose of fed STZ rats

For STZ rats, the first test procedure was performed on day 7 of the experiment. Blood was collected via the saphenous vein for the 0 time point and

immediately following, either a buckwheat concentrate or a placebo was administered intragastrically to fed rats (Figure 6-1A). Blood samples were also collected at 30, 60, 90, and 120 minutes following treatment administration. The total volume of blood samples collected was less than 10% of blood volume of rats.

Effect of buckwheat concentrate on glucose tolerance in fasted STZ rats

For the second test procedure (on day 14 of the experiment), treatments were administered to fasted STZ rats prior to an oral glucose tolerance test (OGTT) (Figure 6-1B). After 4 hours of fasting, rats were given intragastrically either a buckwheat concentrate or placebo. One hour following treatment, blood was collected via the saphenous vein for the 0 time point. Immediately following, rats received 1 g glucose/kg body weight (70% glucose solution) intragastrically. Blood was collected at 30, 60, 90, and 120 minutes after administration of glucose. The total volume of blood samples collected during the OGTT was less than 10% of blood volume of rats. For both tests, blood samples were held on ice until centrifuged to obtain serum. Serum samples were stored at –20°C until analysis.

Analysis of serum glucose

Glucose in the serum was assessed in triplicate using an enzymatic colorimetric kit (Procedure #315, Sigma Chemical Co., St. Louis, MO). Method details are described in Appendix A.

Statistical analysis

Statistical significance between buckwheat and placebo groups was determined by Student's t test using SAS Statistical Software (v. 8.2, SAS Institute Inc., Cary, NC). For effects over time within each treatment group, repeated measures ANOVA was used. Data are expressed as the mean ± SE.

6.4 Results

Composition of buckwheat concentrate

The protein, mineral, and carbohydrate composition of the BWC is shown in Table 6-1. Soluble carbohydrates comprised 12% of the BWC and included D-CI, *myo*-inositol, sucrose, and fagopyritols. The majority of carbohydrates found in the concentrate were from sucrose and fagopyritols. Protein content of the buckwheat concentrate was 5%. The various minerals found in the BWC were present at a concentration of 0.4%. The remainder of the BWC was liquid.

Effect of buckwheat concentrate on glucose tolerance in normal rats

Administration of the low-dose BWC to normal rats 2 hours prior to an IPGTT resulted in serum glucose concentrations that were 17% lower 30 minutes after glucose administration compared to rats given the low-dose placebo (Figure 6-2).

Effect of buckwheat concentrate on serum glucose of fed STZ rats

The low dose BWC group had a 14-16% decrease in serum glucose concentrations 60 to 120 minutes after treatment administration (Figure 6-3). In contrast, rats given the low dose placebo had 4-10% increased serum glucose concentrations after 60 to 120 minutes (Figure 6-3). The high dose BWC also reduced serum glucose concentrations in fed STZ rats by 12% after 90 minutes and by 19% after 120 minutes (Figure 6-4). In rats given the high dose placebo, serum glucose concentrations were similar to baseline values after 60 to 120 minutes (Figure 6-4).

Effect of buckwheat concentrate on glucose tolerance in fasted STZ rats

Administration of the BWC to fasted rats prior to an oral glucose tolerance test did not affect serum glucose concentrations. The mean serum glucose concentrations of both the low dose (Figure 6-5) and high dose (Figure 6-6) BWC-treated rats were lower than the placebo-treated rats, but the difference was not statistically significant.

Table 6-1. Composition of buckwheat concentrates.[1]

Component	Contribution
Carbohydrates (%)	
D-*chiro*-inositol	0.2
myo-inositol	0.1
Sucrose	6.0
Fagopyritols	5.7
Protein (%)	5.0
Minerals (ppm)	
Calcium	130
Iron	2
Magnesium	900
Manganese	2
Phosphorous	2800
Selenium	26
Zinc	60

[1]Amount in high-dose buckwheat concentrate (BWC). For the low-dose BWC, the high-dose BWC was diluted 2-fold so that equal volumes of the concentrate were administered to rats for the two different dose amounts (10 or 20 mg D-CI/kg body weight). The components of the BWC were distributed in liquid.

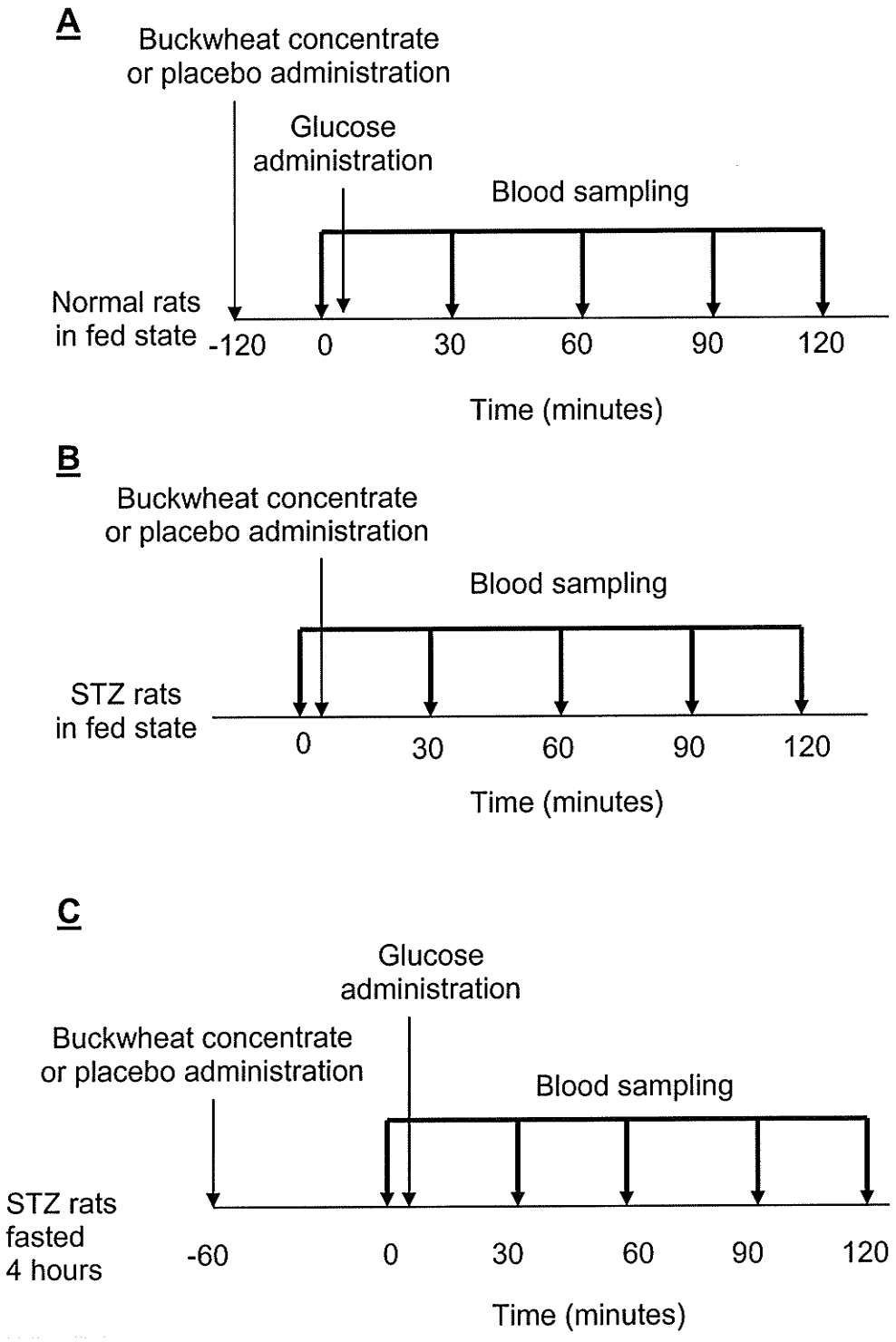

Figure 6-1. Experimental design for (A) intraperitoneal glucose tolerance test 2 hours following an acute dose of a buckwheat concentrate (BWC) or placebo given to normal rats, (B) Fed state response to an acute dose of a BWC or placebo in STZ rats, and (C) Oral glucose tolerance test 1 hour following an acute dose of a BWC or placebo given to fasted STZ rats.

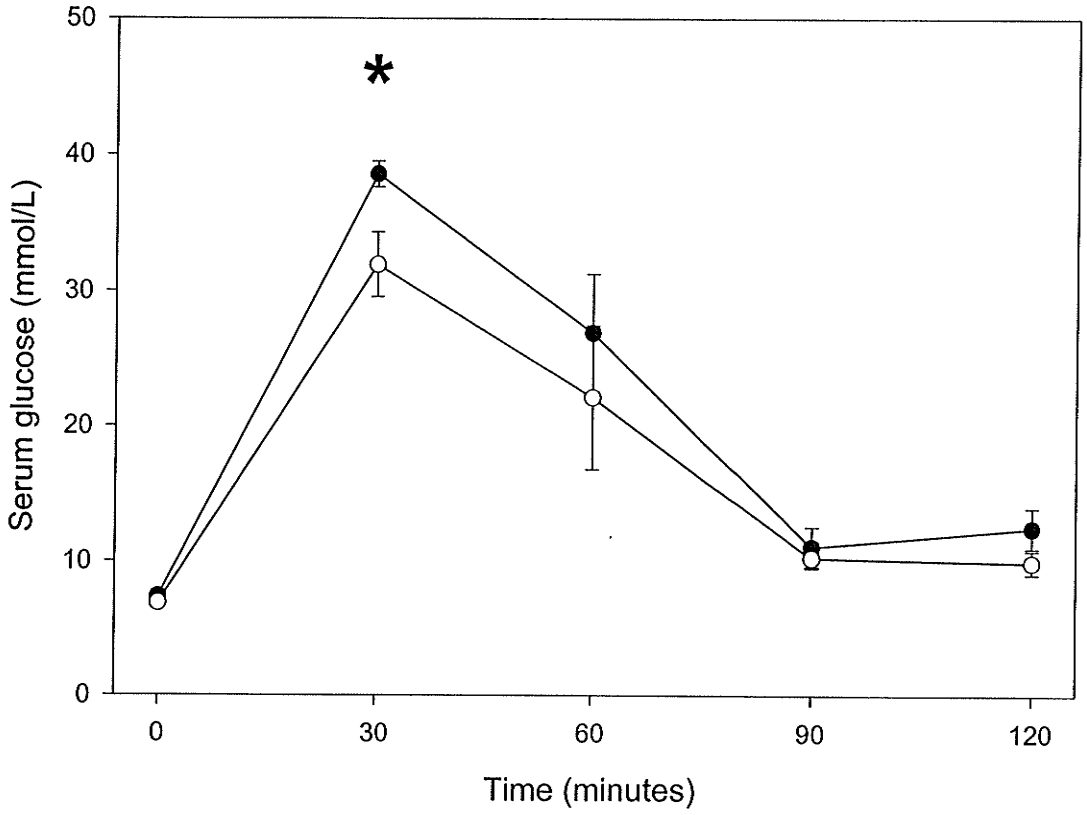

Figure 6-2. Serum glucose concentrations during an intraperitoneal glucose tolerance test given two hours following administration of a low dose buckwheat concentrate (BWC) (10 mg D-*chiro*-inositol/kg body weight) or placebo in normal rats. Solid circles represent the placebo low dose (n=6) and open circles represent the low dose BWC (n=6) groups. An * indicates differences ($p<0.05$) between placebo-treated and buckwheat-treated rats.

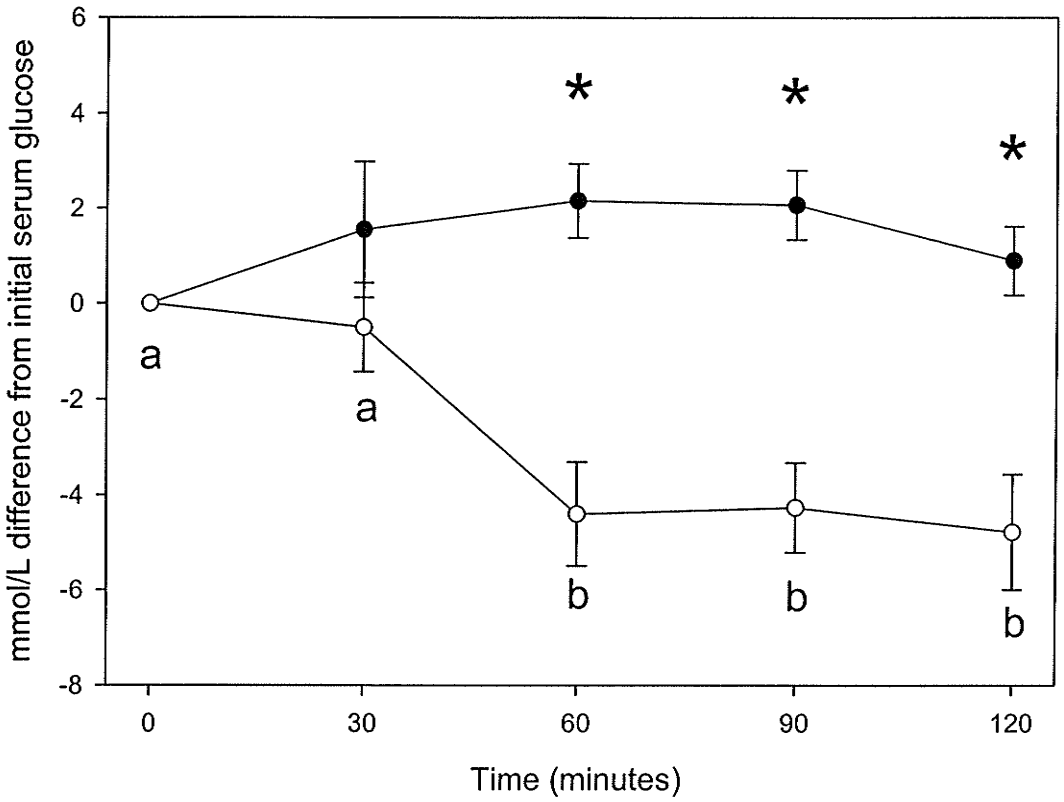

Figure 6-3. Effect of a low dose buckwheat concentrate (BWC) (10 mg D-*chiro*-inositol/kg body weight) or placebo given to STZ rats in the fed state on serum glucose concentrations. Data are expressed as the mmol/L difference from initial serum glucose concentrations (28.4 ± 0.95 mmol/L) for the placebo low dose (solid circles, n=9) and the low dose BWC (open circles, n=8) groups. An * indicates differences ($p<0.001$) between placebo-treated and buckwheat-treated rats. Data points with different letters indicate differences ($p<0.05$) within a group as determined by Duncan's multiple range test.

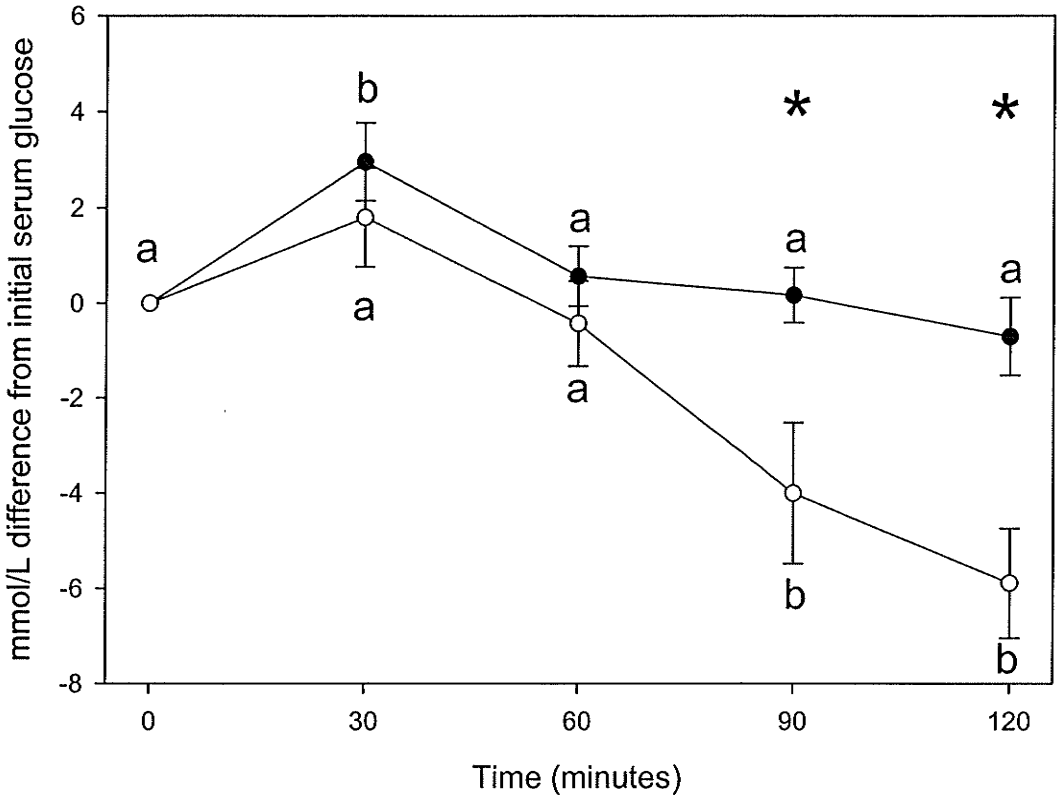

Figure 6-4. Effect of a high dose buckwheat concentrate (BWC) (20 mg D-*chiro*-inositol/kg body weight) or placebo given to STZ rats in the fed state on serum glucose concentrations. Data are expressed as the mmol/L difference from initial serum glucose concentrations (29.6 ± 0.7 mmol/L) for the placebo high dose (solid circles, n=10) and the high dose BWC (open circles, n=9) groups. An * indicates differences ($p<0.05$) between placebo-treated and buckwheat-treated rats. Data points with different letters indicate differences ($p<0.05$) within a group as determined by Duncan's multiple range test.

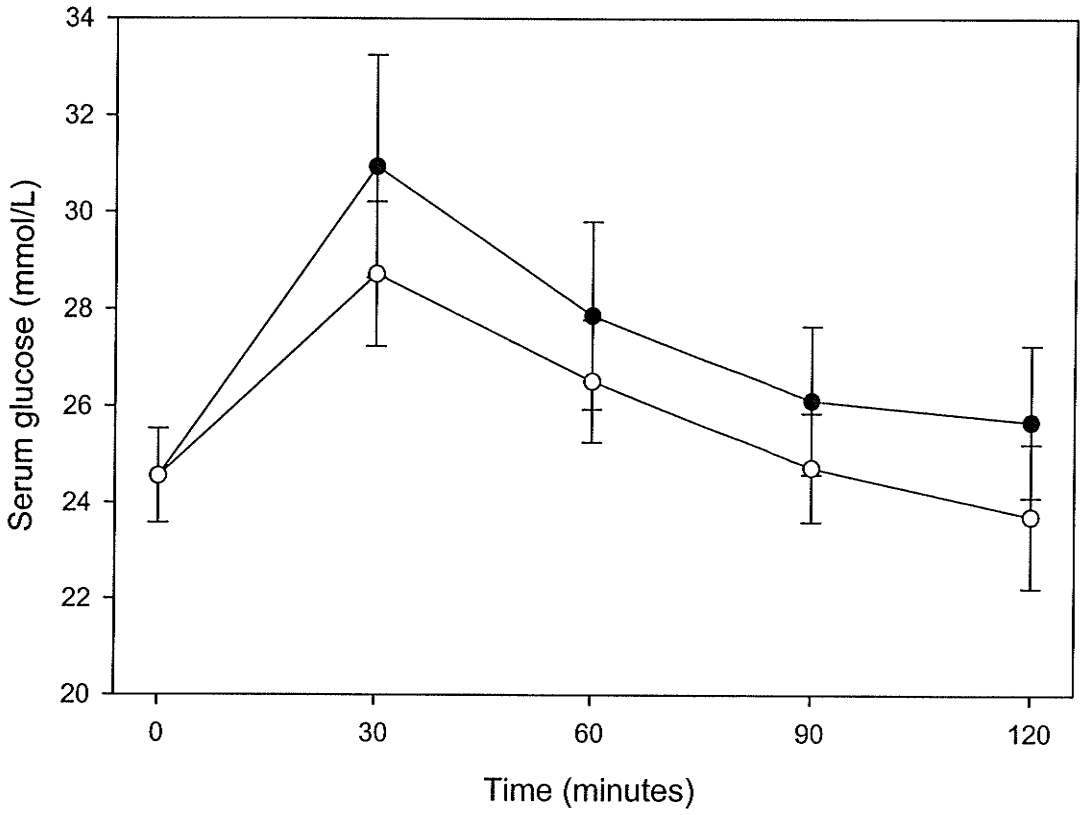

Figure 6-5. Serum glucose concentrations during an oral glucose tolerance test given one hour following administration of a low dose buckwheat concentrate (BWC) (10 mg D-*chiro*-inositol/kg body weight) or placebo in fasted STZ rats. Solid circles represent the placebo low dose (n=9); open circles represent the low dose BWC (n=8) groups.

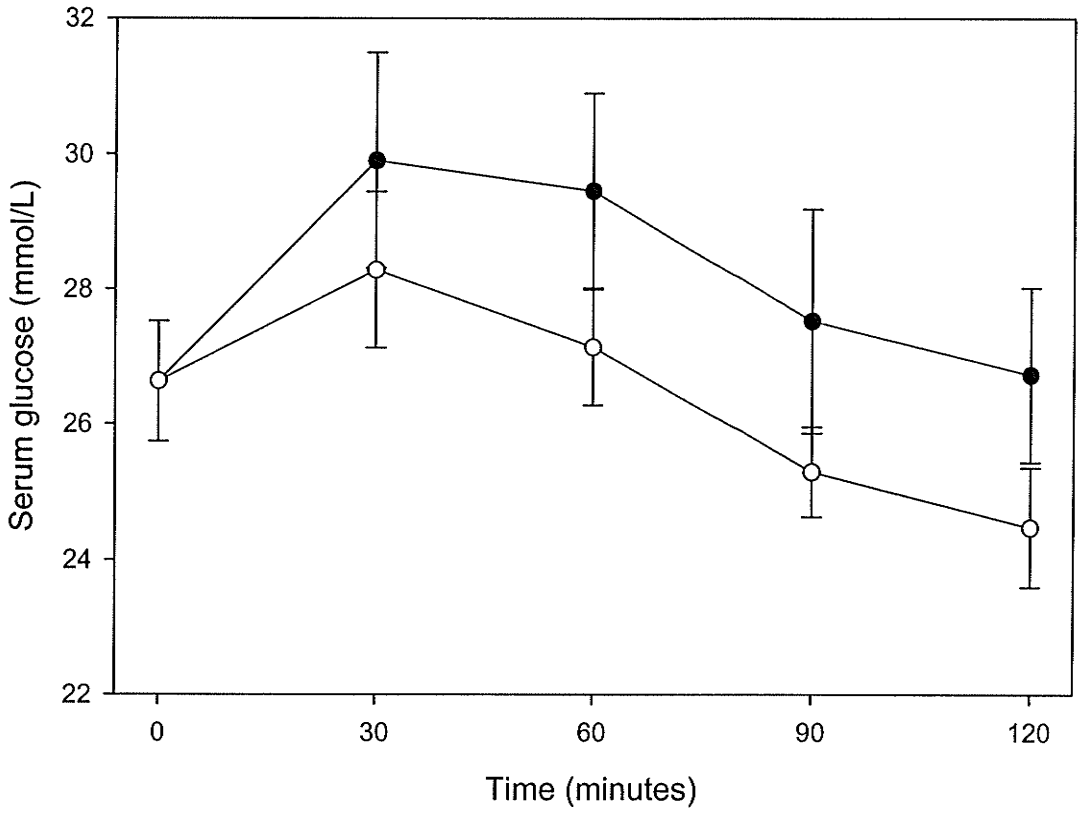

Figure 6-6. Serum glucose concentrations during an oral glucose tolerance test given one hour following administration of a high dose buckwheat concentrate (BWC) (20 mg D-*chiro*-inositol/kg body weight) or placebo in fasted STZ rats. Solid circles represent the placebo high dose (n=10); open circles represent the high dose BWC (n=9) groups.

6.5 Discussion

The major finding of the present study was that a single oral dose of a BWC was effective in lowering elevated serum glucose concentrations in fed STZ rats. In fed STZ rats, both doses (10 and 20 mg D-CI/kg body weight) of the BWC were effective in lowering serum glucose concentrations by 12-19% at 90 and 120 minutes after treatment administration. Similar results were demonstrated when chemically synthesized D-CI was administered to STZ rats. Fonteles *et al* (2000) reported that a single dose of D-CI (15 mg/kg) injected into the jugular vein promoted a 21% decrease in plasma glucose of STZ rats, which was different from the control rats at 80, 100, and 120 minutes after administration. The glucose lowering effect of the BWC demonstrated in the present study is of a similar magnitude to synthesized D-CI, suggesting that D-CI in the buckwheat concentrate is primarily responsible for the observed effects.

In addition to D-CI, the BWC contained MI, also identified as a component of an IPG with insulin-mimetic effects. However, previous work suggests that administration of MI has no effects on plasma glucose concentrations. For example, dietary supplementation with 1.0% MI for 14 days had no effects on hyperglycemia of STZ rats (Greene *et al*, 1975). Besides free D-CI, the BWC used in the present study contained fagopyritols. The role of these D-CI derivatives is not known, however, the degree of glucose-lowering from the BWC was similar to previous reports using synthetic D-CI in amounts equivalent to those present in the BWC. Thus, the contribution of fagopyritols to the glucose lowering effects of the BWC observed in the present study appear to be minimal.

Future research may elucidate a beneficial role for fagopyritols and phytochemicals present in the BWC in other aspects of health and disease.

The mechanism by which administration of D-CI acts to lower plasma glucose is unknown. Ortmeyer *et al* (1993) and Fonteles *et al* (2000) suggested that acute administration of D-CI may act to lower plasma glucose by being incorporated into a mediator precursor. Sanchez-Arias *et al* (1992) demonstrated that STZ rats have impaired GPI-dependent insulin signaling. Isolated hepatocytes from STZ rats had lower amounts of GPI compared to control rats. STZ-induced diabetes also blocked the hydrolysis of GPI in response to insulin and markedly reduced the uptake of IPG. We have reported that urinary D-CI excretion is elevated 336–fold in STZ rats compared to normal rats (Kawa *et al*, 2003). This pattern of inositol excretion may be related to altered GPI-IPG signaling system. It is possible that administration of D-CI corrects the GPI-dependent signaling defect of STZ rats. STZ rats are a model of type 1 diabetes mellitus (DM-1), characterized by hyperglycemia and hypoinsulinemia. Insulin deficiency in DM-1 leads to a decrease in glucose utilization by the liver, muscle, and adipose tissue and an increase in hepatic glucose production (Alemzadeh *et al*, 2002). The antihyperglycemic effect of D-CI may result from inhibition of hepatic glucose output or enhanced glucose transport, glucose utilization, glucose disposal or glycogen synthesis. The mechanism by which administration of D-CI lowers serum glucose concentrations still needs to be established.

The results from this study suggest that an acute dose of the BWC is only effective under specific conditions. Administration of the BWC promoted a decrease in serum glucose concentrations when STZ rats were in the fed state, but did not improve glucose tolerance of fasted STZ rats. To date, this is the first study to report the acute effects of D-CI, in the form of the BWC, given to fasted STZ rats prior to an OGTT (Figure 6-5 and 6-6). In the present study, the BWC was effective despite subsequent glucose administration in normal rats. Similarly, Ortmeyer *et al* (1993) demonstrated that in normal rats, synthetic D-CI administered intragastrically 2 hours before an intraperitoneal glucose load (4 g/kg) produced a 30-50% decrease in plasma glucose. It is possible that administration of glucose to a diabetic animal may compromise the effects of D-CI. Shaskin *et al* (1997) reported that the activity of the IPG containing D-CI increased following glucose ingestion in healthy men whereas no difference in IPG activity was observed in men with type 2 diabetes. Determining the mechanism by which D-CI and the BWC lowers serum glucose in the fed state may also elucidate why the BWC is ineffective in fasted rats given a glucose load.

Findings from this study demonstrate that a BWC is an effective source of D-CI for lowering serum glucose concentrations in rats. Buckwheat can provide a concentrated source of D-CI in the food supply and therefore may be useful in the treatment of diabetes.

7. Buckwheat concentrate stimulates phosphorylation of p70S6 kinase by mitogen-activated protein kinase in H4IIE cells.

Kawa J, Wright B, Taylor C, Przybylski R, Zahradka P.

7.1 Abstract

We previously reported that a buckwheat concentrate (BWC) lowered serum glucose in a diabetic animal model. Insulin acts to lower blood glucose and maintain euglycemia via activation of various cell signal transduction pathways. The BWC contains D-*chiro*-inositol (D-CI) and *myo*-inositol (MI), possible insulin-mimetic compounds. The purpose of the present study was to investigate the insulin-mimetic activities of the BWC on cell signal transduction pathways using H4IIE cells, and to determine the contribution of D-CI and MI to the observed effects. The main finding of this research was that the BWC effectively stimulated phosphorylation of mitogen-activated protein kinase (MAPK) via phospholipase D (PLD) and src. The BWC also stimulated phosphorylation of $p70^{S6K}$ on Thr^{421}, which is a target of MAPK. In contrast, D-CI and MI did not activate these signal transduction proteins. Therefore, we concluded that BWC has insulin-mimetic effects in H4IIE cells, however, D-CI and MI were not the active components responsible for the observed effects.

7.2 Introduction

Recent evidence suggests that buckwheat has antihyperglycemic effects probably due to compounds present in buckwheat that are also found in insulin mimetic inositol phosphoglycan (IPG) molecules. In response to insulin, IPGs are released from glycosylphosphatidylinositols in cell membranes and are incorporated into cells where they can affect enzymes implicated in insulin action (Larner *et al*, 1988, 1989). The insulin-like activities of isolated IPGs and their chemically synthesized analogues have been widely investigated and are summarized in recent reviews (Field,1997; Jones and Varela-Nieto, 1998,1999).

Buckwheat contains relatively high amounts of free D-*chiro*-inositol (D-CI), *myo*-inositol (MI) and galactosyl derivatives of D-CI known as fagopyritols (Horbowicz and Obendorf, 1994). Both D-CI and MI have been identified as components of IPG molecules. We previously demonstrated that oral administration of a buckwheat concentrate (BWC), containing D-CI, MI, and fagopyritols, significantly lowered blood glucose in STZ rats (Kawa *et al*, 2003; Chapter 5). Two doses of the BWC were effective for lowering serum glucose concentrations by 12-19% at 90 and 120 minutes after intragastric administration in fed STZ rats, however, the mechanism for the glucose-lowering effects was not determined.

Insulin affects glucose, protein and lipid metabolism by binding to its receptor, which subsequently activates various cell signal transduction pathways that mediate the biological actions of insulin. Among the downstream effects of insulin binding is regulation of the glucose transporter GLUT4 that results in

increased glucose uptake in skeletal muscle and adipose tissue (Wood and Trayhurn, 2003). Insulin also increases glucose utilization by affecting key enzymes involved in glucose metabolic pathways primarily at the level of transcription, and insulin increases glucose storage by activating signal transduction proteins in pathways leading to glycogen synthesis. Other downstream effects of insulin receptor activation include increased lipogenesis and increased protein synthesis. The MI containing IPG has demonstrated insulin-mimetic effects including *in vitro* stimulation of glucose transport, GLUT-4 translocation, glycogen synthesis, lipogenesis and protein synthesis. Stimulation of PI3K, MAPK and inhibition of GSK-3 are within pathways typically linked to insulin, and have also been documented for the MI containing IPG (Field, 1997; Jones and Varela-Nieto, 1998, 1999). Although it has been less studied, the D-CI containing IPG has also demonstrated insulin mimetic effects *in vitro* including the activation of key protein phosphatases in pathways known to be stimulated by insulin. In particular, the IPG containing D-CI has demonstrated the activation of pyruvate dehydrogenase (PDH) phosphatase and glycogen synthase phosphatase (Field, 1997; Jones and Varela-Nieto, 1998, 1999). These enzymes play a key role in the regulation of glucose disposal by oxidative metabolism (glycolysis) and by the non-oxidative route of storage by glycogen synthesis, respectively (Kunjara *et al*, 1999).

The purpose of the present study was to evaluate potential mechanisms for the glucose-lowering effects of the BWC in relation to insulin and IPGs. H4IIE hepatoma cells were used in the present study to characterize the role of the

BWC in insulin-dependent signaling systems. The liver plays a central role in glucose metabolism and insulin is able to stimulate glucose metabolic pathways in this cell line. Thus, the objectives of this research were to determine the effects of BWC versus insulin on phosphorylation of signal transduction proteins and to determine which cell signaling pathways were activated by BWC in H4IIE rat hepatoma cells.

7.3 Materials and Methods

Materials

Tissue culture media, antibiotics, fetal bovine serum (FBS) and Nunc tissue culture plates were purchased from Invitrogen. Insulin (Sigma) was dissolved in water and added directly to cells at a concentration of 250 nM unless otherwise indicated. The BWC was prepared and analyzed as described by Kawa *et al* (2003) (Chapter 6, Section 6.3). Two μL of the BWC were added directly to cells unless otherwise indicated. This amount was chosen on the basis of equivalence to insulin for its western blot band intensity of P-MAPK stimulation. *Myo*-inositol (Sigma) and D-*chiro*-inositol (Industrial Research Limited) were dissolved in water at an equal concentration to the amounts present in the BWC (Appendix B, Table B-17) and 2 μL of each solution (0.1% v/v final concentration) was added directly to cells.

The polyclonal antibodies against phospho-Akt (Ser^{473}), phospho-AMPK (Ser^{108}), phospho-ELK-1 (Ser^{383}), phospho-GSK3 ($Ser^{21/9}$), phospho-Insulin receptor (Tyr^{1146}), p42/44 MAPK, phospho-p42/44 MAPK (Thr^{202}/Tyr^{204}), phospho-$p70^{S6K}$ (Thr^{389} and Thr^{421}), phospho-PDK-1 (Ser^{241}), phospho-PRK1 (Thr^{778}), phospho-Raf (Ser^{259}), phospho-STAT3 (Tyr^{705}) and phospho-S6 ribosomal protein ($Ser^{235/236}$) were purchased from Cell Signaling. The polyclonal antibody against phospho-Src (Tyr^{527}) was obtained from Biosource and phospho-Insulin receptor substrate-I (Tyr^{941}) was obtained from Oncogene. The HRP-coupled anti-(rabbit IgG) was purchased from Bio-Rad.

Compounds used as selective inhibitors of signal transduction were obtained from Calbiochem (Pertussis toxin, Raf kinase inhibitor, Go 7874 and AG1024), New England Biolabs (PD98059), Biomol Inc (LY294002, AG490, Go 6976, PP1, Rottlerin, U73122) and Sigma (Brefeldin A, 1-butanol, and 2-butanol). Inhibitors details are provided in Table 7-1.

The BCA protein assay kit was supplied by Pierce. Bovine serum albumin (BSA, fraction V) was purchased from Roche. Polyvinylidenedifluoride (PVDF) membranes were from Millipore. The ECL chemiluminescent detection system was provided by Amersham Biosciences. 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.

Cell culture

Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548) were cultured as previously described (Yau *et al*, 1998). Briefly, cells were maintained in α-modified Eagle's media containing 10% FBS, 2 mM glutamine, 25 $\mu g \cdot mL^{-1}$ 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.

Treatments and sample preparation

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. Inhibitors were added 10 minutes before the stimulating agents except for pertussis toxin which was added 4 hours prior. The cells in 12-well culture dishes were incubated with stimulating agents for 6 minutes and rinsed with phosphate buffered saline (PBS; 0.9% NaCl, 0.1 M Na_3PO_4, pH 7.1).

Western blotting

Cellular protein extracts were prepared by addition of 200 µL 2x SDS/gel loading buffer (0.125 M Tris pH 6.8, 2% SDS, 10% glycerol) to H4IIE cells in 12-well culture dishes. The samples were briefly sonicated, and protein content measured using the BCA protein assay kit. Aliquots containing equal amounts of protein were mixed with bromophenol blue (0.5% (w/v) final concentration) and 2-mercaptoethanol (5% (v/v) final concentration), heated at 95°C and loaded onto 7.5% or 10% polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membrane and the membranes subsequently blocked in 3% (w/v) BSA-TBST. Primary antibodies (diluted 1:1000 in 3% (w/v) BSA) were incubated with the membrane for 60 min at 22°C. Membranes were subsequently washed four times and incubated for an additional 60 min at 18-22°C in 1% (w/v) BSA-TBST with diluted HRP-coupled secondary antibody (1:10 000). HRP was detected using the ECL chemiluminescent system after the membranes had been washed four times. Further details of the methods are provided in Appendix A.

Quantification of data obtained on film (Kodak X-omat) was accomplished with a GS800 imaging densitometer (Bio-Rad Laboratories, Missisauga, ON) under nonsaturating conditions with local background subtraction. Although

multiple exposures were acquired to ensure the absence of film saturation, the experimental figures typically show longer exposures selected specifically for visual presentation and not necessarily used for data analysis. Graphical data are presented as means ± SE for a minimum of three replicate samples.

Statistical analyses

Statistical significance between treatment groups was determined using a mixed model analysis with a random intercept for the repetitions (SAS v.8.2, SAS Institute, Cary, NC) and by estimate statements for individual comparisons of treatments versus the control. Differences were accepted as significant at $p<0.05$. Data are expressed as the mean ± SE.

7.4 Results

In a previous study, BWC effectively lowered serum glucose concentrations in STZ rats (Kawa *et al*, 2003; Chapter 6). Buckwheat contains D-CI and MI, components of putative insulin mediators with demonstrated insulin-like activity, which suggests that the BWC also acts as an insulin mimetic. In order to determine the mechanism by which the BWC lowers serum glucose, we examined its effects on key processes activated by insulin in H4IIE cells. Based on our current understanding of insulin-dependent signal transduction (Figure 7-1), the effects of the BWC on protein phosphorylation within the various insulin-stimulated cell signaling pathways were investigated in the present study. The proteins indicated in boldface within the grey circles in Figure 7-1 represent those tested for stimulation by the BWC.

Effects of the buckwheat concentrate on protein phosphorylation

As shown in Figure 7-2, some signal transduction proteins are phosphorylated in response to insulin, while treatment with the BWC, D-CI or MI does not result in their stimulation. These include InsR, IRS-1, Akt, Src, STAT3 and GSK3. In contrast, both insulin and the BWC increased the phosphorylation of MAPK (Figure 7-3), while neither D-CI nor MI increased phosphorylation of this protein. To determine if these results were unique to the H4IIE cells, we also evaluated the effects of the BWC on phosphorylation of MAPK in FAO hepatoma cells. In FAO cells, the BWC and insulin also stimulated MAPK phosphorylation whereas D-CI and MI did not (data provided in Appendix D).

The concentration-dependent phosphorylation of MAPK by the BWC is shown in Figure 7-4. Phosphorylation of MAPK was effectively increased by 0.5 μL to 20 μL of the BWC. To determine if insulin and the BWC affect MAPK to a similar degree, the effect of combining these agents was tested (Figure 7-5). The data indicate that the effects of insulin and the BWC on MAPK are additive.

Figure 7-6 shows proteins that are not activated by any of the treatments. Among these are PDK, PRK1/2, RAF and ELK1. Interestingly, both D-CI and MI reduce phosphorylation of AMPK, as shown in Figure 7-7 although the effects were not statistically significant ($p=0.06$ and $p=0.075$, respectively) versus the control.

Cell signaling pathways of the BWC

To assess the intracellular signaling pathways that contribute to the actions of the BWC, we evaluated the effects of inhibiting phospholipase D (PLD), Src kinase (Src), and Arf3 on the ability of INS and the BWC to subsequently stimulate selected signal transduction proteins. Figure 7-8A shows the band intensities for phosphorylation of InsR and MAPK by insulin and the BWC when PLD, Src, and Arf3 were inhibited. Phosphorylation of InsR was stimulated by insulin only, and PLD, Src, and Arf3 were not required (Figure 7-8B). The BWC did not stimulate phosphorylation of InsR, the same as the result presented in Figure 7-2A. As indicated in Figure 7-8C, both insulin and the BWC stimulate MAPK phosphorylation. PLD (butanol sensitive) is required for stimulation of MAPK by insulin and the BWC, whereas Src (PP1 sensitive) is

required for stimulation of MAPK by the BWC only. Arf3 (brefeldin sensitive) is not involved in this process.

The effects of other inhibitors on insulin and BWC stimulated phosphorylation of MAPK, PDK1 and Raf were also determined. These inhibitors are listed in Table 7-1. As expected, insulin and BWC-dependent stimulation of MAPK phosphorylation were inhibited by PD98059 (data provided in Appendix D) whereas no other inhibitors had any effects. Phosphorylation of PDK1 and Raf was not stimulated by either insulin or the BWC, and subsequently none of the inhibitors had any effects on these proteins.

We also assessed the effects of the BWC versus insulin on phosphorylation of proteins downstream from MAPK. As shown in Figure 7-9, the BWC stimulated 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 $p70^{S6K}$, requires dual $p70^{S6K}$ phosphorylation. Figure 7-10A and B shows that stimulation of $p70^{S6K}$ (Thr^{421}) by both insulin and the BWC is MAPK dependent (PD98059 sensitive), but is independent of PI3K (LY294002 sensitive). In contrast, insulin-dependent stimulation of $p70^{S6K}$ (Thr^{389}) phosphorylation requires activation of PI3K but is independent of MAPK (Figure 7-10C). A summary of these results is shown in Figure 7-11. Other individual pathways leading from the insulin receptor could also be incorporated into this scheme (as shown in Figure 7-1), but the lack of effect by the BWC suggests they are of no relevance for further investigation.

Table 7-1. Inhibitors of signal transduction proteins in the insulin signaling pathway.

Compound	Inhibits phosphorylation of	Working Concentration	Reference
PD98059	MAPK	10^{-5} M	Yau *et al*, 1999
Brefeldin A	phospholipases	50 μg/mL	Li *et al*, 1998
1-butanol	PLD1	0.3%	Morton *et al*, 1995
PP1	src	10^{-5} M	Zahradka *et al*, 2004
U73122	PLC	10^{-5} M	Smith *et al*, 1990
Raf kinase 1 inhibitor	Raf1	10^{-7} M	Lackey *et al*, 2000
AG490	Stat3	10^{-5} M	Sharfe and Dadi, 1995
LY294002	PI3K	10^{-5} M	Saward and Zahradka, 1997
AG1024	Insulin receptor	0.5×10^{-5} M	Zahradka *et al*, 2004
Pertussis toxin	G-protein	5 μL/mL	Hsia *et al*, 1984
Go7874	PKA	10^{-6} M	Kleinschroth *et al*, 1995
Go6976	PKA	10^{-6} M	Martiny-Baron *et al*, 1993
Rottlerin	PKC δ	10^{-6} M	Gschwendt *et al*, 1994

Inhibitors were prepared in DMSO (Sigma) unless otherwise indicated.

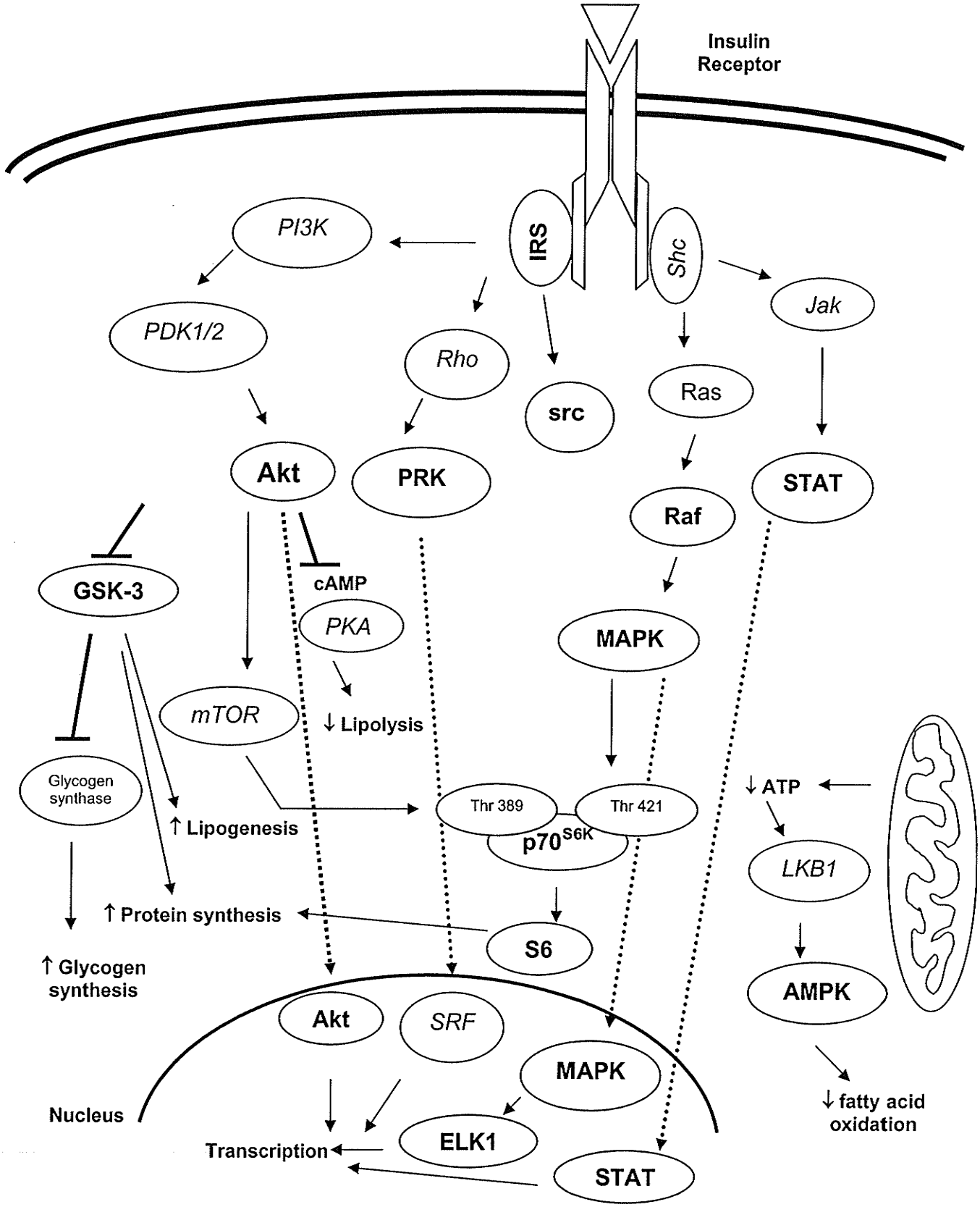

Figure 7-1. Cell signal transduction pathways activated by insulin. Signal transduction proteins in boldface within grey circles were tested in the present study for stimulation by the buckwheat concentrate.

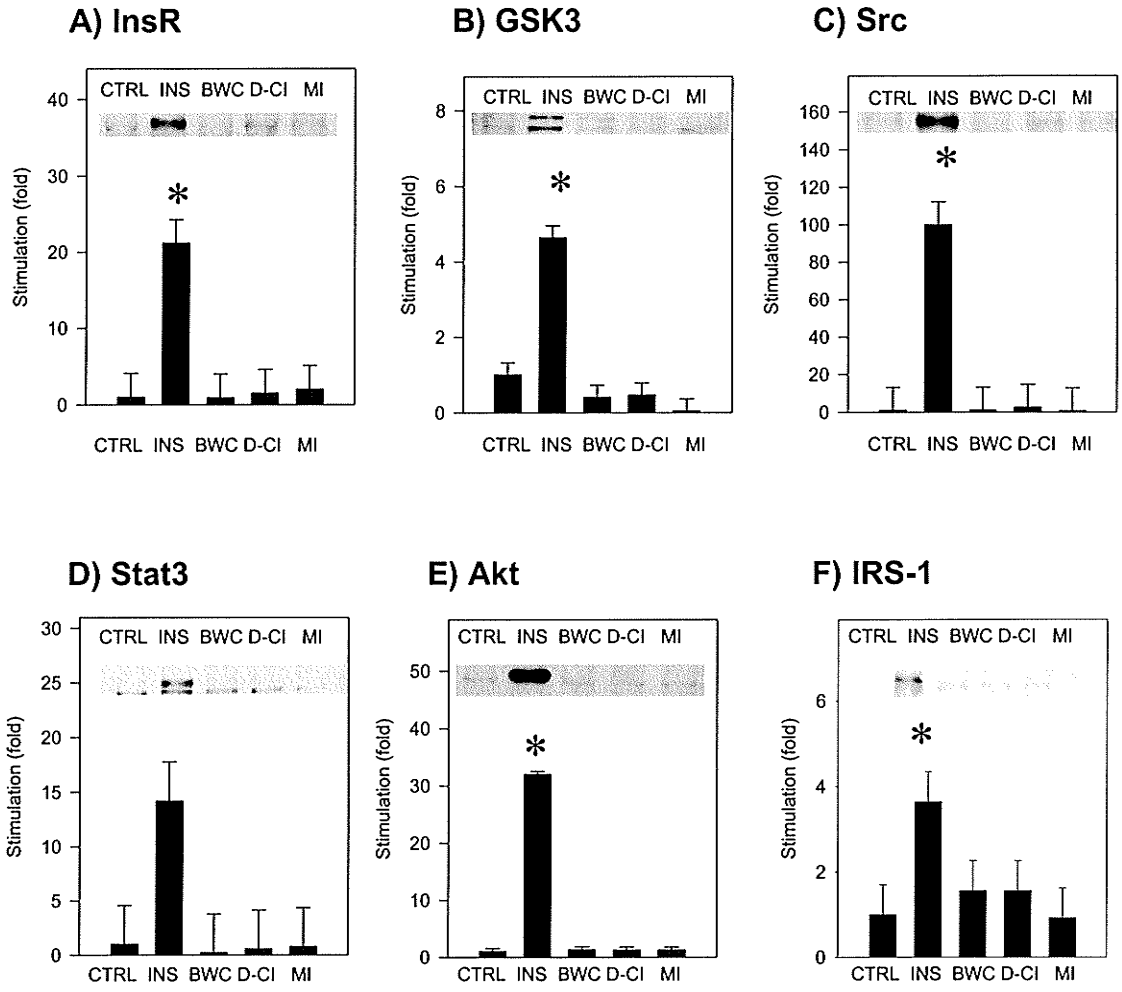

Figure 7-2. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of select signal transduction proteins assessed by Western blot analysis. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Each experiment was replicated three times. Band intensities on each Western blot were quantified by scanning densitometry and plotted as means ± SE (n=3). 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 $p=0.06$.

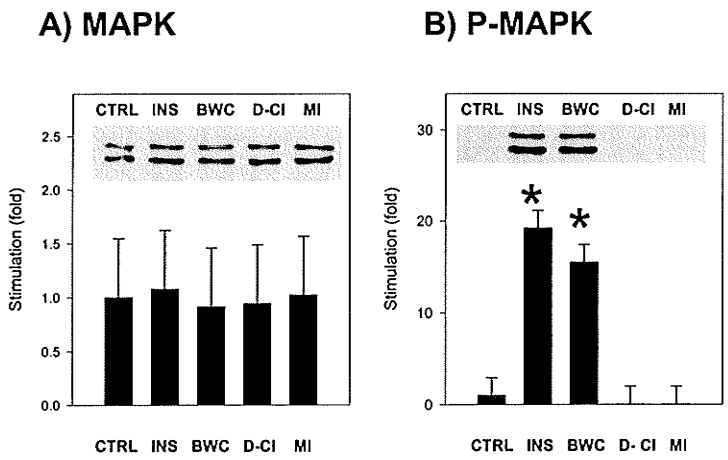

Figure 7-3. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on stimulation of A) mitogen-activated protein kinase (MAPK) and B) phosphorylated (P)-MAPK assessed by Western blot analysis. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SE (n=3). Significant differences ($p<0.05$) between treatments versus the control (*) are indicated.

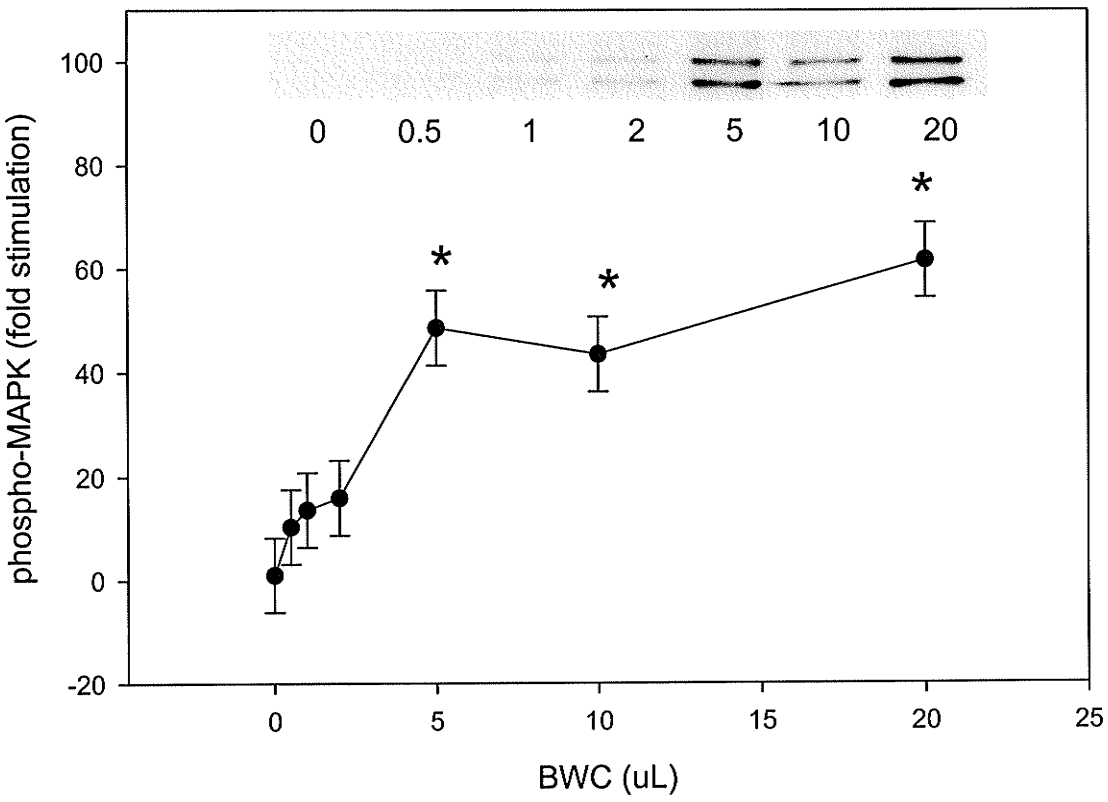

Figure 7-4. The concentration-dependent effects of the buckwheat concentrate (BWC) on mitogen activated protein kinase (MAPK) phosphorylation assessed by Western blot analysis. H4IIE cells were treated with increasing amounts of the BWC individually for 6 minutes. Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SE (n=3). Significant differences ($p<0.05$) between each BWC amount versus the control (*) are indicated.

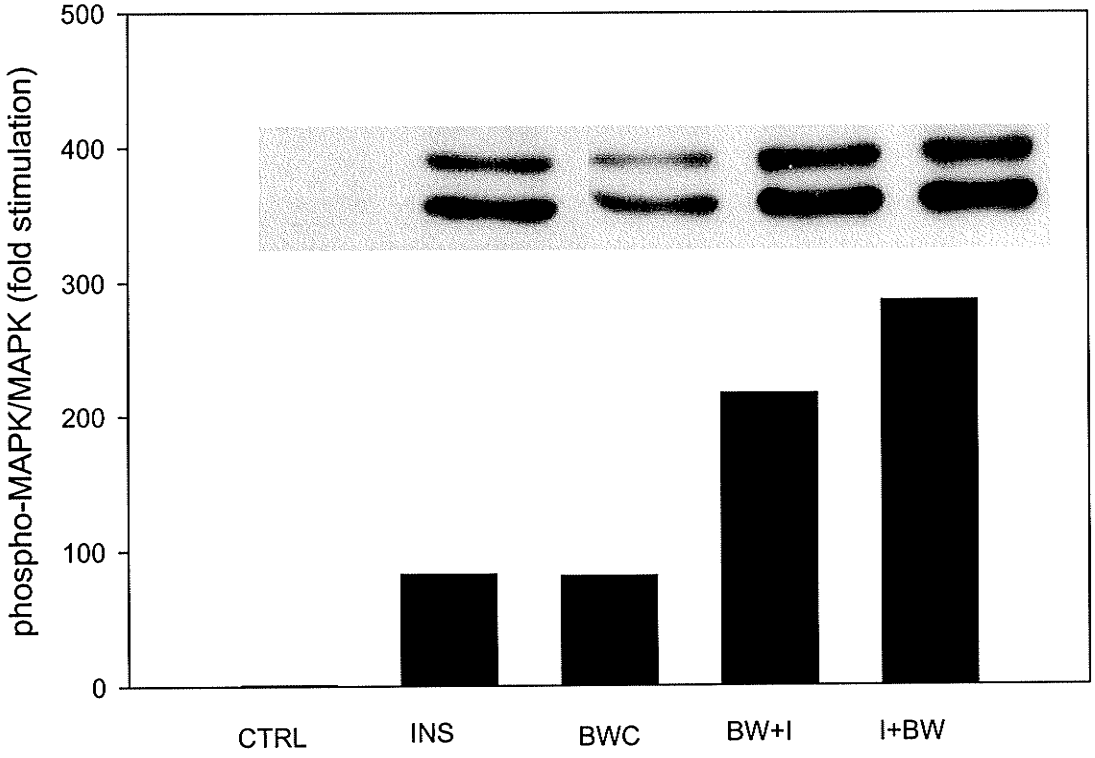

Figure 7-5. The effects of insulin (INS) and the buckwheat concentrate (BWC) on phosphorylation of mitogen-activated protein kinase (MAPK) assessed by Western blot analysis. H4IIE cells were treated for 6 minutes with INS, BWC or a combination of both where either BWC (BW+I) or INS (I+BW) was added first. Untreated cells served as the control (CTRL). Band intensities were quantified by scanning densitometry. This figure illustrates data from one experiment.

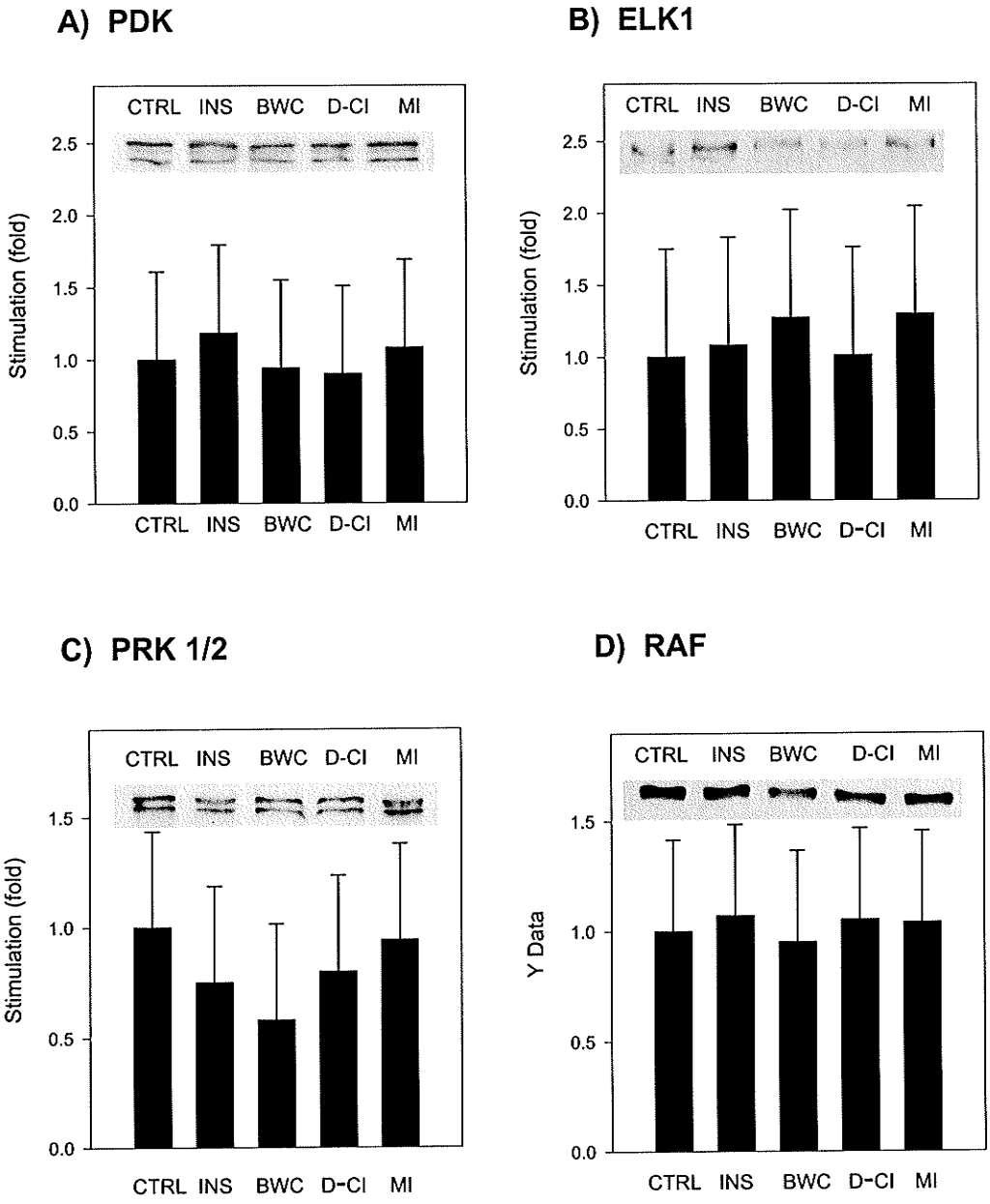

Figure 7-6. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of select signal transduction proteins assessed by Western blot analysis. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SE (n=3). There were no significant differences between any of the treatments versus the control.

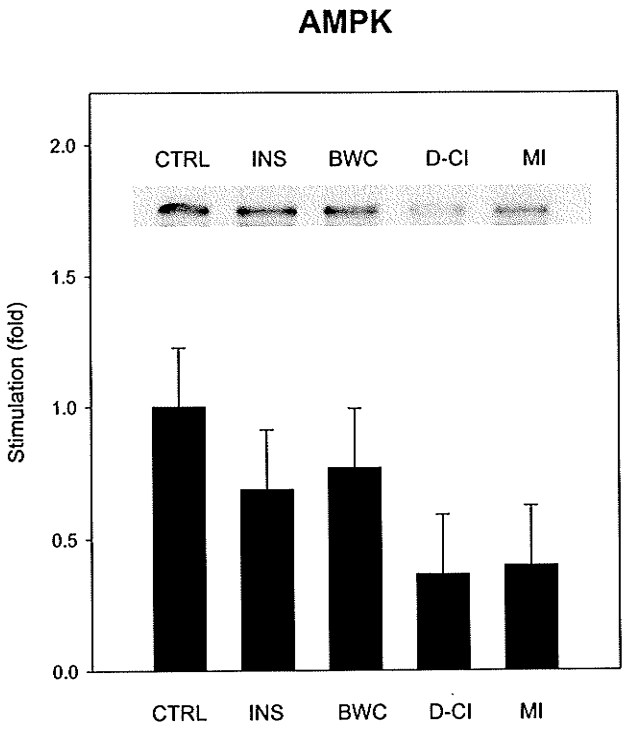

Figure 7-7. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of AMP-dependent protein kinase (AMPK) assessed by Western blot analysis. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SE (n=3). The statistical analysis provided p-values for the difference between the CTRL versus D-CI and MI at p=0.06 and p=0.075 level of significance, respectively.

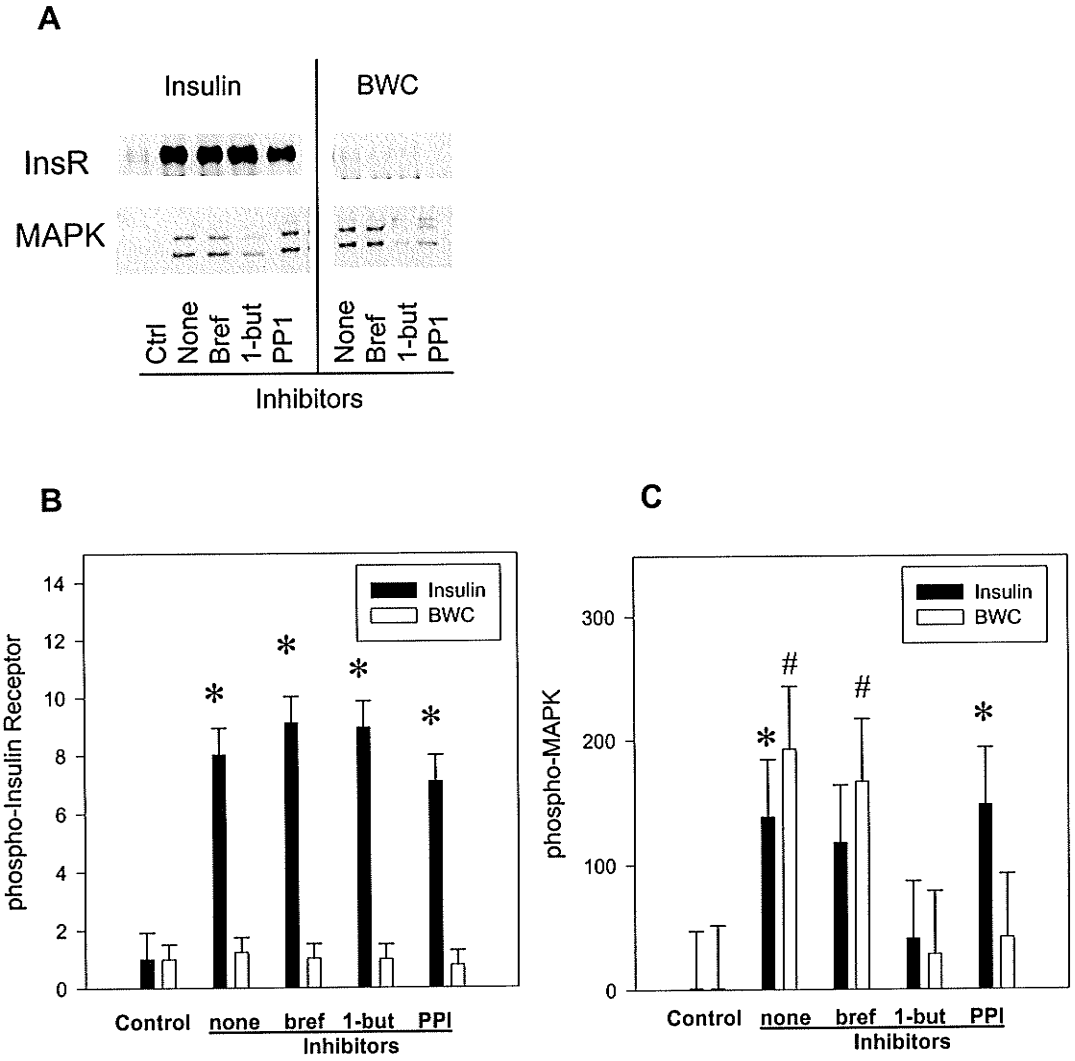

Figure 7-8. The effects of inhibition of phospholipase D (butanol sensitive), Src kinase (PP-1 sensitive), and arf3 (brefeldin sensitive) on buckwheat concentrate (BWC) and insulin stimulated phosphorylation of A) insulin receptor (InsR) and mitogen-activated protein kinase (MAPK) assessed by Western blot analysis. Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with either insulin or the BWC for 6 minutes. Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and plotted as means ± SE (n=3). The effects of inhibitors on BWC and insulin stimulated phosphorylation of B) InsR and C) MAPK are shown. 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. The comparison for insulin versus insulin plus each inhibitor did not indicate differences. In contrast, the BWC was significantly higher than the BWC+1-but and the BWC+PP1 ($p<0.05$).

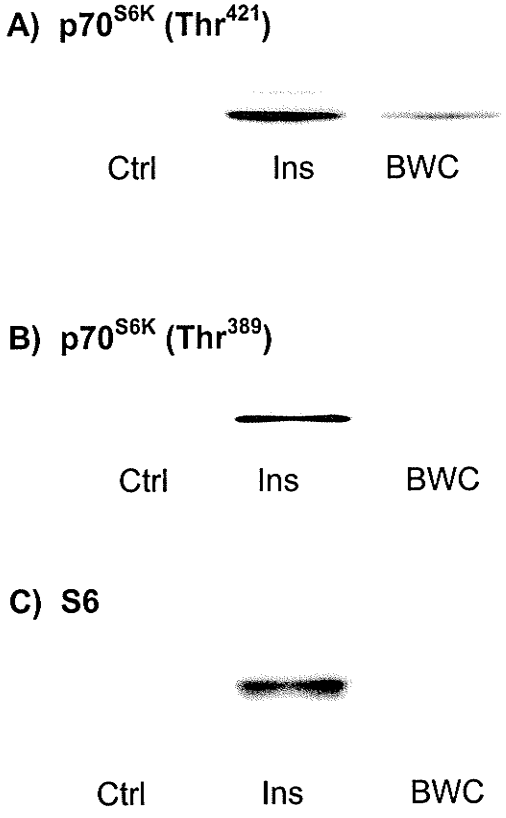

Figure 7-9. The effects of insulin (INS) and the buckwheat concentrate (BWC) on phosphorylation of A) $p70^{S6K}$ (Thr^{421}), B) $p70^{S6K}$ (Thr^{389}) and C) ribosomal protein S6 assessed by Western blot analysis. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Representative blots are shown.

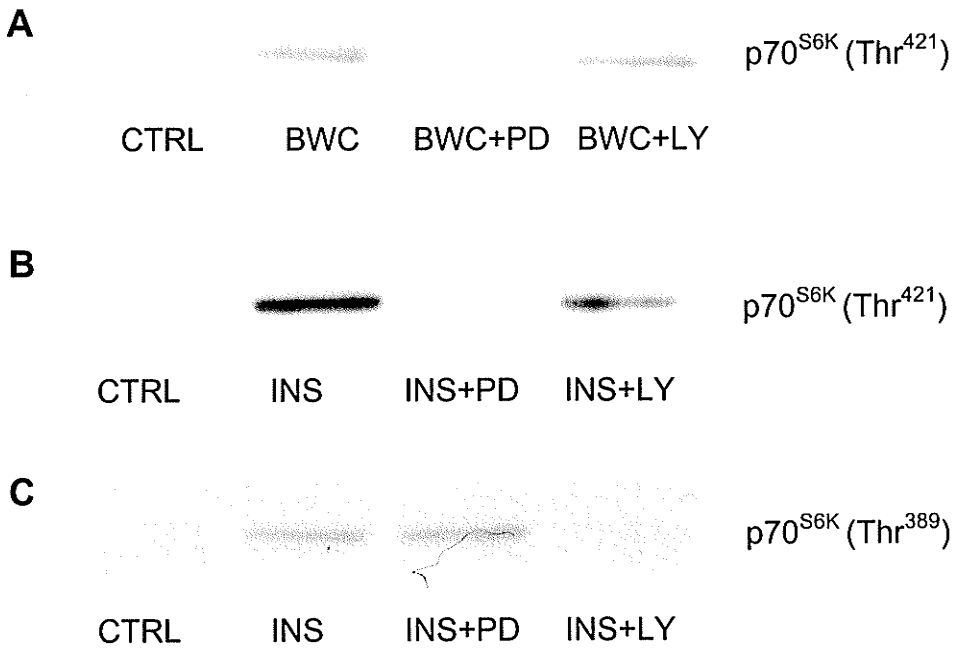

Figure 7-10. The effects of inhibition of MAPK (PD98059 (PD)-sensitive) and PI3K (LY294002 (LY)-sensitive) on A) buckwheat concentrate (BWC)-stimulated phosphorylation of $p70^{S6K}$ (Thr^{421}), B) insulin (INS)-stimulated phosphorylation of $p70^{S6K}$ (Thr^{421}) and C) INS-stimulated phosphorylation of $p70^{S6K}$ (Thr^{389}). Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with either INS or the BWC for 6 minutes, with untreated cells serving as the control (CTRL). Representative blots are shown.

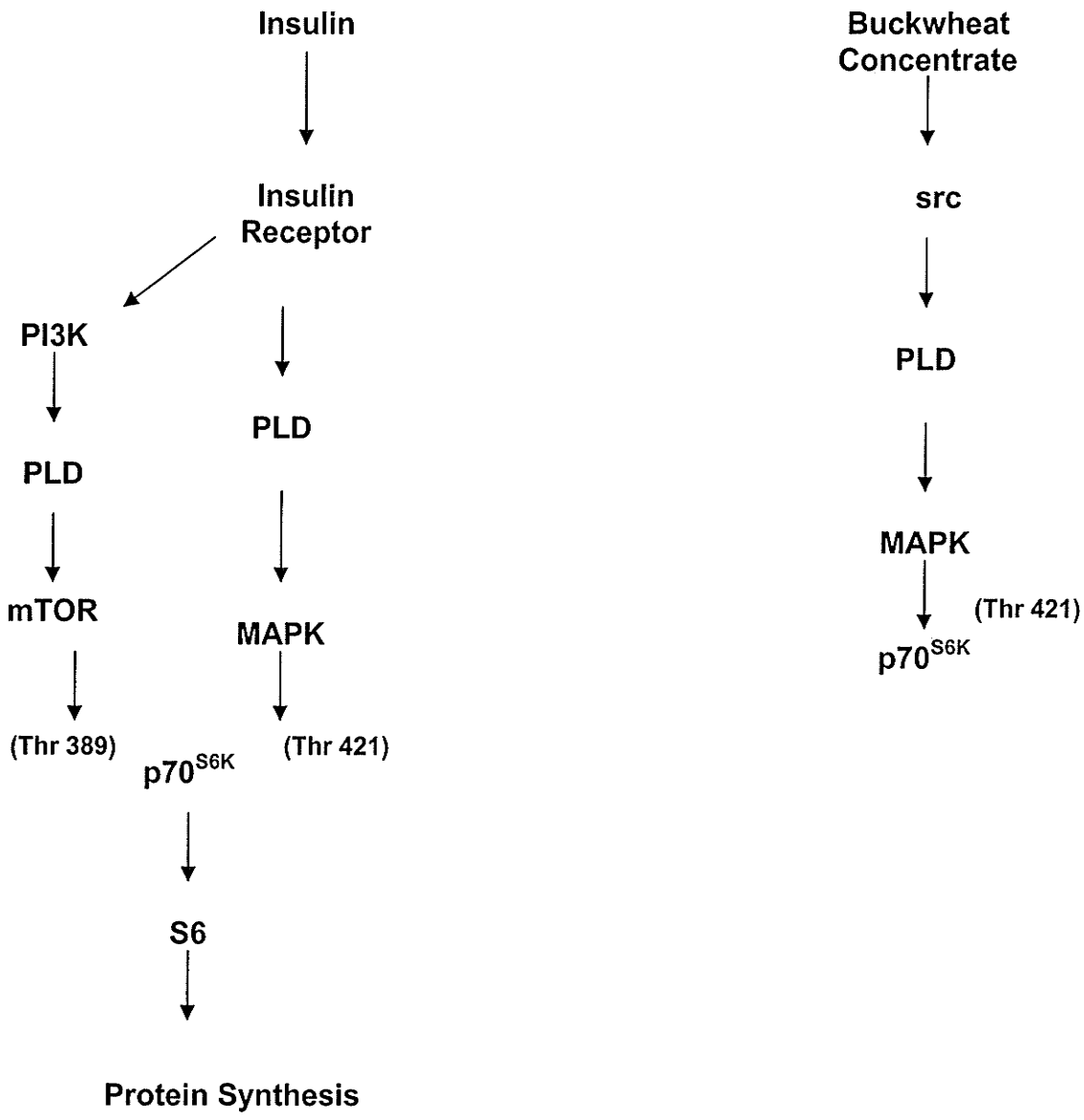

Figure 7-11. Summary of the effects of insulin and the buckwheat concentrate on phosphorylation of signal transduction proteins in H4IIE cells.

7. 5 Discussion

We previously demonstrated that oral administration of a BWC significantly lowered blood glucose concentrations in STZ rats (Kawa *et al*, 2003; Chapter 6). In the present study, we investigated the effects of the BWC versus insulin on phosphorylation of signal transduction proteins and characterized the cell signaling pathways of the BWC, using H4IIE rat hepatoma cells. The results presented here indicate that the BWC activates MAPK phosphorylation via PLD. The BWC also stimulates phosphorylation of $p70^{S6K}$ on Thr^{421}, which is a target of MAPK.

Insulin activates numerous cell signaling pathways upon binding to its receptor. Among the downstream effects resulting from these pathways are increased glucose uptake, utilization and storage, as well as decreased glucose production and release, increased lipogenesis and protein synthesis (Figure 7-1). Insulin mimetic compounds can also stimulate phosphorylation of proteins within these pathways, thus inducing similar biological effects. Some current antidiabetic treatments act in this manner to stimulate glucose uptake, directly affecting insulin signaling response cascades and/or decreasing hepatic glucose production (Meriden, 2004). IPGs containing D-CI and MI have also demonstrated insulin mimetic effects including increased glucose uptake, utilization and storage (Field, 1997; Jones and Varela-Nieto, 1998, 1999). Since the BWC contains D-CI and MI, we hypothesized that the previously reported glucose-lowering effects of the BWC were due to the presence of these insulin-

mimetic compounds. However, the results of the present study indicate a separate cell signaling pathway for the BWC versus D-CI and MI.

Insulin binding promotes the activation and autophosphorylation of its receptor which then catalyses the phosphorylation of several substrates including IRS (Saltiel and Pessen, 2002; Saltiel and Kahn, 2002; Burks and White, 2001; Summers *et al*, 1999). Tyrosine-phosphorylated IRS then activates PI3K which has a major role in insulin function, mainly via the activation of the Akt. Activated Akt induces glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK-3). Promotion of fatty acid synthesis and inhibition of lipolysis are also downstream of activated Akt. Protein synthesis is also downstream from $p70^{S6K}$ which phosphorylates ribosomal protein S6. The Ras/MAPK and Akt/mTOR pathways both participate in this pathway by mediating the phosphorylation of distinct Thr residues on $p70^{S6K}$. Insulin mediates transcription in the cell nucleus through the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, the Akt cascade, as well as by activation of the Ras/mitogen activated protein kinase (MAPK) pathway. Various enzymes involved in glucose metabolism are also regulated by insulin's effect on gene transcription (Saltiel and Pessen, 2002; Saltiel and Kahn, 2002; Burks and White, 2001; Summers *et al*, 1999).

The significance of MAPK phosphorylation in response to treatment with the BWC in the present study could be related to gene transcription. Modification of MAPK results in its activation and subsequent translocation into the nucleus, where it mediates the phosphorylation of specific transcription factors (Figure 7-

1). Thus, it is possible that the mechanism for previously observed glucose lowering effects of the BWC is related to gene transcription downstream from MAPK phosphorylation. Hepatic glucokinase is an enzyme that converts glucose to a form that is both trapped in the cell and capable of being further metabolized (Shimomura *et al*, 2000; King, 2002). Insulin increases expression of this enzyme as well as other enzymes and proteins involved in glucose metabolism. Thus, it is possible that the BWC has a similar effect.

As shown in Figure 7-8C, both PLD (butanol sensitive) and src (PP1 sensitive) are required for BWC-dependent stimulation of MAPK phosphorylation, whereas only PLD is required for insulin-dependent stimulation of MAPK phosphorylation. PLD is a phospholipid-degrading enzyme that is recognized for its abilitiy to generate biologically active products which are assumed to play important functions in cell regulation (Liscovitch *et al*, 2000). The interaction of extracellular-signal molecules with cell-surface receptors including insulin often activates a PLD-mediated hydrolysis of phospholipids (Liscovitch *et al*, 2000). In fact, the release of IPGs from cell membranes in response to insulin is considered to be mediated by PLD (Jones and Varela-Nieto, 1999). It is possible the the BWC is also involved in this response.

The lack of effect of PP1 on insulin-dependent stimulation of MAPK suggests that insulin and the BWC operate through distinct mechanisms to activate PLD. Of interest is the fact that the BWC requires src for phosphorylation of MAPK (Figure 7-8C) but is not able to stimulate src phosphorylation (Figure 7-2C). Src belongs to the src family of non-receptor

tyrosine kinases whose nine members also include LYN, FYN and LCK among others (Bevilaqua *et al*, 2005). Since PP1 inhibits all members of the src family, it may be another member of the src kinase family besides src that is required for BWC-stimulated phosphorylation of MAPK. This would explain why src is required for MAPK activation by BWC despite the fact the BWC does not stimulate phosphorylation of src. Arf3 (brefeldin sensitive) is typically depicted as linking the insulin receptor to PLD (Li *et al*, 2003), however, in the present study its inhibition had no effect on BWC or insulin-stimulated activation of MAPK.

$p70^{S6K}$ is downstream from MAPK and is required for cell growth (Gabele *et al*, 2005). $p70^{S6K}$ also phosphorylates the ribosomal protein S6, which is involved primarily in protein synthesis. The BWC stimulated phosphorylation of $p70^{S6K}$ (Thr^{421}) but not $p70^{S6K}$ (Thr^{389}), unlike insulin which stimulated phosphorylation of both $p70^{S6K}$ residues. Although the BWC did not stimulate S6 phosphorylation, insulin did, indicating the requirement for phosphorylation of both the Thr^{421} and Thr^{389} residues on $p70^{S6K}$ for subsequent phosphorylation of S6. The significance of the BWC-stimulated phosphorylation of $p70^{S6K}$ (Thr^{421}) may be related to cell growth or another unknown downstream effect.

Insulin was effective for stimulating phosphorylation of InsR, IRS-1, GSK3, STAT3, src, and Akt unlike the BWC, D-CI, and MI which did not increase phosphorylation of these proteins (Figure 7-2). These results indicate that the BWC (D-CI and MI) does not affect glucose metabolism with regards to glycogen synthesis via the Akt/GSK-3 pathway or transcription via the Jak/STAT, PI3K/Akt and PRK/SRF pathways in H4IIE cells. Thus, the BWC, D-CI, and MI should not

be able to stimulate lipogenesis and protein synthesis via Akt, or inhibit lipolysis via Akt in this cell type. Results from the present study also indicate that certain signal transduction proteins within insulin signaling pathways are constitutively phosphorylated in H4IIE cells. Among these are PDK, PRK1/2, Raf and ELK1.

In an attempt to elucidate the active component/s of the BWC responsible for the observed effects, we also evaluated the effects of free D-CI and MI on phosphorylation of signal transduction proteins. The insulin-mimetic effects of free D-CI and MI have not been evaluated 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 IPGs (Ortmeyer *et al*, 1993; Fonteles *et al*, 2000). Unlike the BWC in the present study, D-CI and MI were unable to affect phosphorylation of MAPK or $p70^{S6K}$ (Thr^{421}). Although D-CI and MI did not stimulate the phosphorylation of any signal transduction proteins in the present study, both inositol isomers reduced AMPK phosphorylation. AMPK is postulated to decrease acetyl-CoA carboxylase activity and therefore decrease fatty acid oxidation (Sakamoto *et al*, 2005). The lack of insulin-mimetic effects of free D-CI and MI in comparision to previous reports with IPGs may be related to the cell type used in the present study and/or could potentially be due to a lack of incorporation of these compounds into IPG molecules.

Nonetheless, the results presented here suggest that another component in buckwheat besides D-CI or MI is responsible for the insulin-mimetic effects, or there is possibly an interaction of these and/or compounds within the BWC that is

responsible for the previously observed antihyperglycemic effects. Buckwheat contains galactosyl derivatives of D-CI known as fagopyritols. Fagopyritols are relatively unique to buckwheat and are present at high levels (Horbowicz and Obendorf, 1994). Glucose lowering effects of whole buckwheat have been reported in previous studies although the active components were not identified (Biljani *et al*, 1985; Lu *et al*, 1992; Wang *et al*, 1992). Fagopyritols have not yet been evaluated for their glucose lowering effects but it is possible that they may have some effect on hyperglycemia. Various polyphenolic flavonoid compounds including rutin, quercetin, catechins, epicatechin, hyperoside and proanthocyanidins have also been identified in buckwheat (Watanabe, 1998; Quettier-Deleu *et al*, 2000; Holasova, 2002; Yokazawa, 2002; Fabjan, 2003). Lignans are another class of polyphenolic compound reported to be present in buckwheat (Setchell, 1995). It is possible that one or more of these compounds is responsible for the insulin-mimetic effects of the BWC as several bioflavonoids and phenolic compounds have been reported to improve hyperglycemia in diabetes by affecting glucose transport, insulin-like properties, and insulin-receptor function (Jung *et al*, 2004)

Results from the present study demonstrate that the BWC has insulin-mimetic effects in H4IIE cells, with respect to phosphorylation of MAPK and p70S6K (Thr^{421}) downstream from MAPK and that PLD and src are required. Activation of these cell signal transduction proteins by the BWC may be related to the glucose-lowering effects of BWC previously observed in STZ rats. Both insulin and the BWC require PLD activation for phosphorylation of MAPK,

however, the BWC also requires phosphorylation of src (Figure 7-8C) despite the lack of BWC-stimulated src phosphorylation (Figure 7-2C). The $p70^{S6K}$ protein is downstream from MAPK and its phosphorylation on the Thr^{421} residue was also stimulated by both insulin and the BWC. In contrast, only insulin was effective for stimulation of $p70^{S6K}$ (Thr^{389}) phosphorylation. PI3K is required for $p70^{S6K}$ phosphorylation on the Thr^{389} residue but is independent of the Thr^{421} residue. Phosphorylation of ribosomal protein S6, which leads to protein synthesis, is downstream from phosphorylation of both $p70^{S6K}$ residues (Thr^{421} and Thr^{389}). Insulin requires activation of the insulin receptor for its downstream effects, whereas the BWC has a different entry point. 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.

8. Effects of a buckwheat concentrate on cellular glucose uptake

Kawa J, Wright B, Taylor C, Przybylski R, Zahradka P.

8.1 Abstract

A buckwheat concentrate (BWC) effectively reduced hyperglycemia in rats in a previous study. Blood glucose homeostasis results from a balance between glucose uptake, utilization and storage versus glucose production and output. In the liver, which plays a central role in glucose metabolism, glucose uptake is stimulated by insulin due to increased activity of enzymes involved in glucose utilization and storage. The BWC contains D-*chiro*-inositol (D-CI) and *myo*-inositol (MI), putative insulin-mimetic compounds. In an effort to determine the mechanism for the previously observed glucose lowering effects of the BWC, we investigated the ability of the BWC versus insulin, D-CI, and MI to stimulate glucose uptake in H4IIE cells. In the present study, insulin and D-CI effectively stimulated glucose uptake. A similar effect was observed by MI, although it was not significant versus the control. In contrast, the BWC inhibited basal and insulin-stimulated glucose uptake. Therefore, BWC does not stimulate glucose uptake and thus employs an apparently unique mechanism for lowering blood glucose concentrations. Furthermore, the D-CI and MI present in the BWC were unable to stimulate glucose uptake in H4IIE cells, suggesting that other compounds present in the BWC were responsible for the inhibitory effects that were observed.

8.2 Introduction

In a recent study, a buckwheat extract (BWC) demonstrated antihyperglycemic effects in streptozotocin (STZ) rats (Kawa *et al*, 2003; Chapter 6). Buckwheat contains relatively high amounts of free D-*chiro*-inositol (D-CI), *myo*-inositol (MI) and galactosyl derivatives of D-CI known as fagopyritols (Horbowicz and Obendorf, 1994). Both D-CI and MI have been identified as components of inositol phosphoglycan (IPG) molecules. In response to insulin, IPGs are released from cell membranes and have demonstrated intracellular insulin-mimetic effects (Larner *et al*, 1988). The insulin-like activities of isolated IPGs and their chemically synthesized analogues have been widely investigated and are summarized elsewhere (Field,1997; Jones and Varela-Nieto, 1998,1999).

We previously demonstrated that oral administration of a BWC, containing D-CI, MI, and fagopyritols, was effective for lowering serum glucose concentrations by 12-19% at 90 and 120 minutes after intragastric administration to STZ rats in the fed state (Kawa *et al*, 2003; Chapter 6). The glucose lowering effects in rats of chemically synthesized D-CI in its free form have also been reported (Ortmeyer *et al*, 1993; Fonteles *et al*, 2000). The mechanism for the antihyperglycemic effects of free D-CI and the BWC (containing D-CI) was presumed to be related to the insulin-mimetic activities of IPGs, however, this has not been investigated.

Insulin maintains glucose homeostasis and reduces hyperglycemia by decreasing hepatic glucose production and release, and increasing glucose

uptake, utilization, and storage in body tissues. Insulin directly affects glucose uptake in skeletal muscle and adipose tissue through its regulation of the GLUT4 glucose transporter (Kanzak and Pessin, 2001). The liver is one of the major targets of insulin for glucose uptake, utilization, and storage, but insulin's effects on hepatic glucose uptake are different than in muscle and adipose. In response to insulin, hepatic glucose uptake is dramatically augmented due to increased activity of enzymes involved in glucose utilization and storage via pathways of glycolysis and glycogenesis (King, 2002). This leads to a decrease in the intracellular glucose concentration, and consequently, glucose enters hepatocytes via facilitated diffusion in response to the concentration gradient that forms. In contrast to GLUT4 in skeletal muscle and adipose, the hepatic glucose transporter (GLUT2) is insulin independent (Tirone and Brunicardi, 2001).

Among the reported insulin-mimetic effects of IPGs are activation *in vitro* of enzymes and transcription of genes related to glucose metabolism, stimulation of glycogen synthesis, lipogenesis, and protein synthesis (Field,1997; Jones and Varela-Nieto, 1998,1999). The liver plays a central role in glucose metabolism and these effects of insulin are observed in the liver leading to stimulation of glucose uptake. We hypothesized that the BWC would have insulin mimetic effects in H4IIE rat hepatoma cells and would increase glucose uptake in these cells as a result of increased glucose utilization and storage. Thus, the objectives of the present study were to determine the effects of the BWC, D-CI, and MI versus insulin on glucose uptake using H4IIE cells.

8.3 Materials and Methods

Materials and reagents

Tissue culture media, antibiotics, fetal bovine serum (FBS) and Nunc tissue culture plates were purchased from Gibco-BRL. Insulin (Sigma) was dissolved in water and added directly to cells at a final concentration of 10^{-6} M. The buckwheat extract was prepared and analyzed as previously described (Kawa *et al*, 2003; Chapter 6). Two μL of the buckwheat extract were added directly to cells unless otherwise indicated. This amount was chosen on the basis of equivalence to insulin for its western blot band intensity of P-MAPK stimulation. *Myo*-inositol (Sigma) and D-*chiro*-inositol (Industrial Research Limited) were dissolved in water at an equal concentration to the amounts present in the BWC (Appendix B, Table B-17) and 2 μL of each solution was added directly to cells.

1-butanol was obtained from Sigma. PD98059 was obtained from New England Biolabs. AG1024 was purchased from Calbiochem. Inhibitor details are provided in Table 8-1. ^{3}H-deoxyglucose was purchased from New England Nuclear/Perkin Elmer. General laboratory chemicals were purchased from Sigma. Ultrapure chemicals were obtained from Gibco-BRL, Bio-Rad or Boehringer-Mannheim (Roche).

Cell culture

Rat H4IIE hepatoma cells (American Type Culture Collection, CRL 1548) were cultured as previously described (Yau *et al*, 1998). Briefly, cells were maintained in α-modified Eagle's media containing 10% FBS, 2 mM glutamine,

50 $\mu g \cdot mL^{-1}$ streptomycin and 50 $\mu g \cdot mL^{-1}$ 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 (Yau *et al*, 1998)

Assay of ^{3}H-deoxyglucose uptake in H4IIE cells

^{3}H-deoxyglucose uptake was assayed according to the method of Harrison *et al* (1990). Briefly, quiescent H4IIE cells, in 24-well culture dishes containing 1 mL serum-free medium, were washed three times with physiological salt solution (PSS) (5 M NaCl, 2 M KCl, 1 M Hepes, 1 M $MgSO_4$, 0.2 M Na_2HPO_4, 0.5 M $CaCl_2$, pH 7.4). Cells were placed into 0.5 mL PSS and pre-incubated with inhibitors for 10 minutes prior to the 20 minute stimulation with treatments (insulin, BWC, D-CI, MI). For all experiments, insulin was added at a concentration of 10^{-6} M whereas 2 μL volumes of the BWC, D-CI, or MI (0.4% v/v final concentration) were added to cells unless otherwise indicated in figures. Sugar uptake was initiated by the addition of ^{3}H-deoxyglucose to a final assay concentration of 0.1 mM glucose (1 mCi/mL) and the cells were subsequently incubated for 5 minutes at 37°C. After two rapid washes with ice-cold PSS (0.5-1 mL/well), the cells were solubilized with 0.4 mL of 0.1% SDS, and ^{3}H was detected in 3 mL of scintillant (EcoLume, ICN).

Statistical analyses

Statistical significance between treatments was determined by one-way ANOVA and by Duncan's multiple range test for means testing (SAS v.9.1, SAS

Institute Inc., Cary, NC). Differences were accepted as significant at $p<0.05$. Data are expressed as the mean ± SE.

8.4 Results

A BWC has been shown to lower serum glucose concentrations in STZ rats (Kawa *et al*, 2003; Chapter 6). Since insulin has the same effect, we compared the ability of the BWC, insulin and two insulin-mimetic compounds (D-CI and MI) to stimulate glucose uptake by H4IIE hepatoma cells. Quiescent H4IIE cells were placed into glucose-free medium and stimulated with these agents for 10 minutes. Radiolabelled deoxyglucose was added to the medium and glucose uptake was determined by the detection of ^{3}H in cells.

As shown in Figure 8-1, insulin and D-CI stimulate glucose uptake. While MI also appears to increase glucose uptake, it is not statistically different from untreated cells. In contrast, the BWC has no stimulatory activity and actually appeared to inhibit 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) decreased the basal rate of glucose uptake, although the effect was not significant in this experiment (Figure 8-2). Interestingly, adding the BWC to cells 10 minutes prior to treatment with insulin resulted in inhibition of glucose uptake. This inhibition of insulin-stimulated glucose uptake by the BWC (I+B) is shown in Figure 8-3.

We also investigated the contribution of various signal transduction mediators on insulin-stimulated glucose uptake. As shown in Figure 8-4, inhibiting the phosphorylation of MAPK (PD98059 sensitive) and insulin receptor tyrosine kinase (AG420 sensitive) decreased insulin-stimulated glucose uptake.

Thus, activation of these proteins is required for insulin to stimulate glucose uptake. Inhibiting phospholipase D (butanol sensitive) also decreased insulin-stimulated glucose uptake although the effect was not significant.

Table 8-1. Inhibitors of signal transduction proteins in the pathway of insulin signaling.

Compound	Inhibitor of	Working Concentration	References
PD98059	MAPK	10^{-5} M	Yau *et al*, 1999
1-butanol	PLD1	0.3%	Morton *et al*, 1995
AG1024	Insulin receptor	0.5 x 10^{-5} M	Zahradka *et al*, 2004

Inhibitors were prepared in DMSO (Sigma).

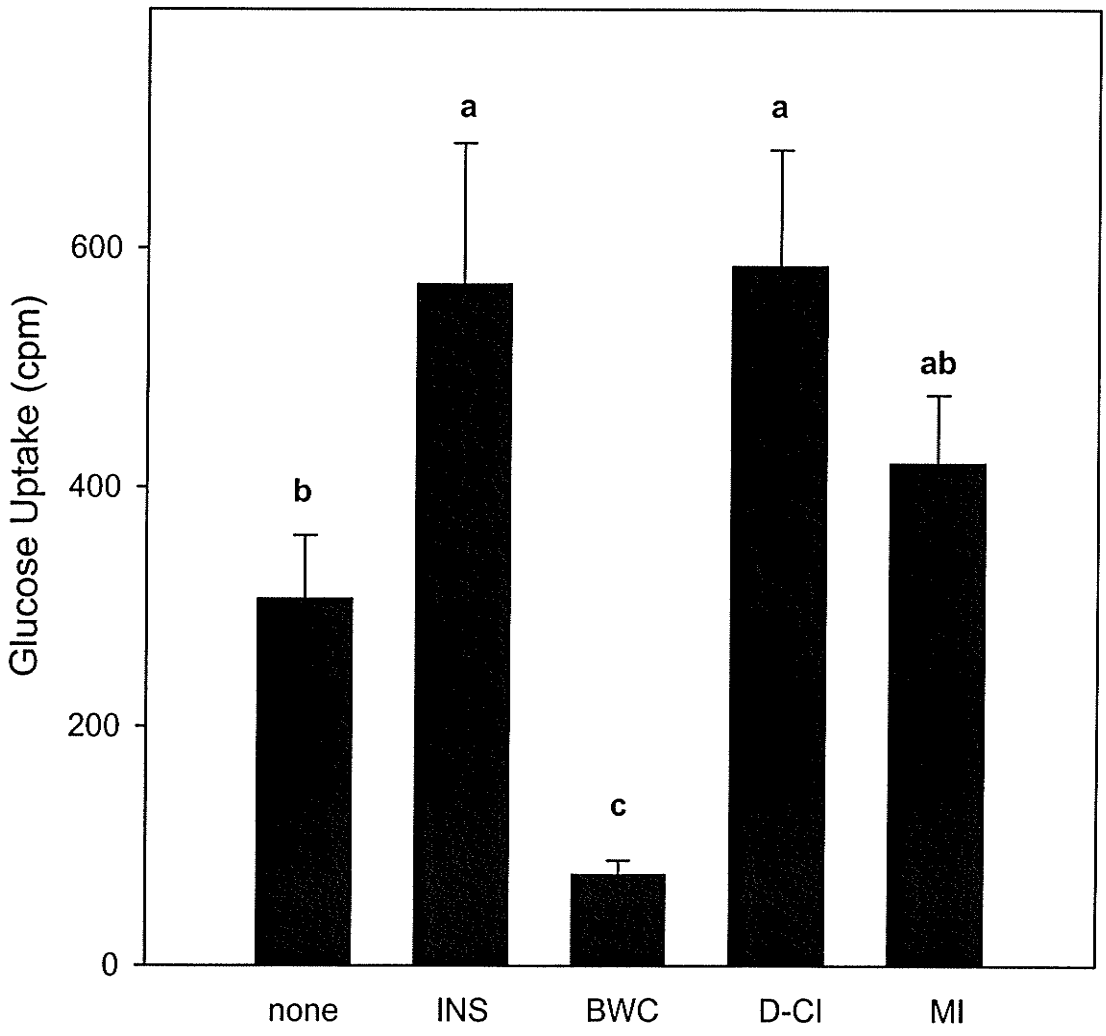

Figure 8-1. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on ^{3}H-deoxy-glucose uptake in H4IIE cells. The data are presented as means ± SE for three independent experiments. Statistical significance ($p<0.05$) was determined by ANOVA and Duncan's multiple range test and bars with different letters are significantly different.

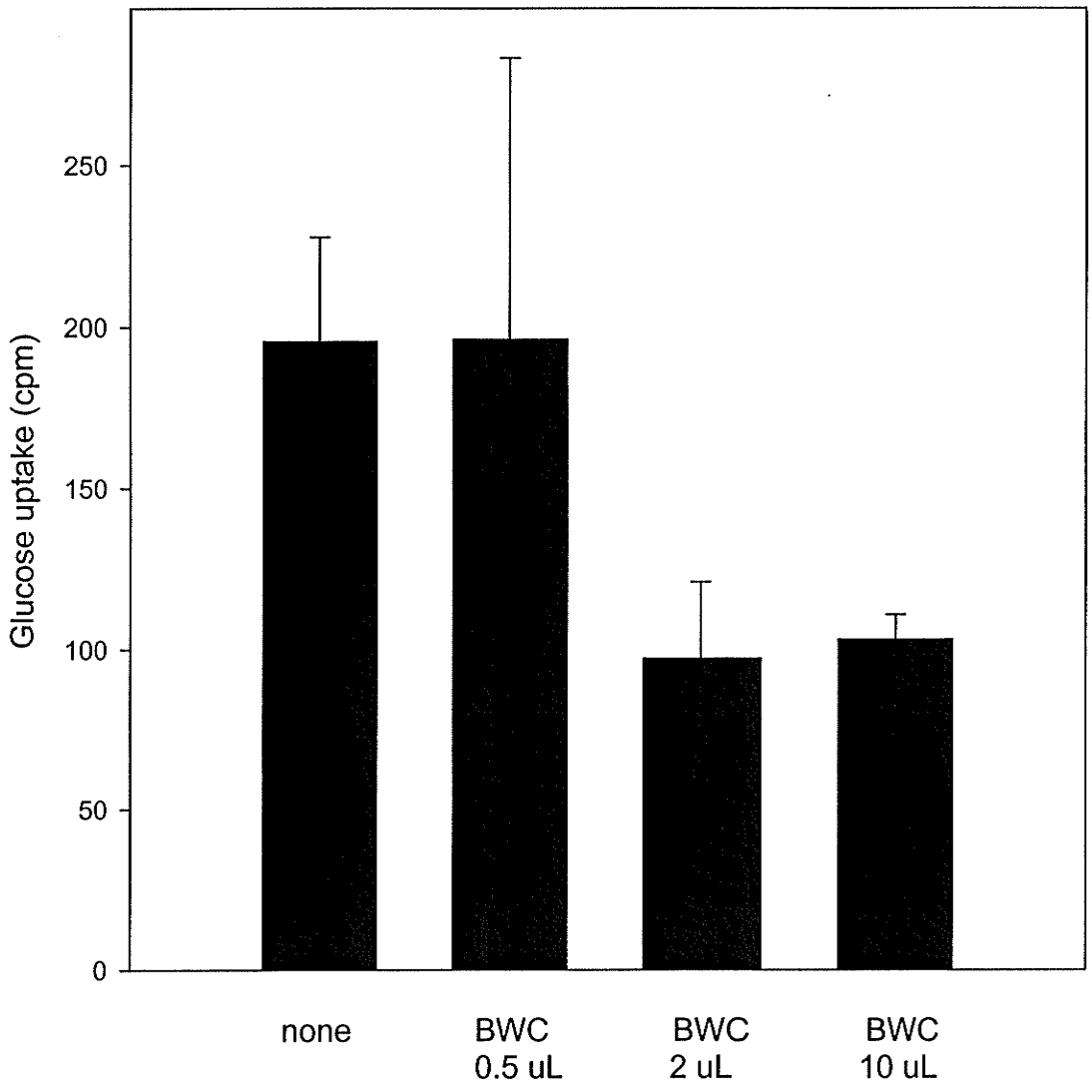

Figure 8-2. The dose-dependent effects of the buckwheat concentrate (BWC) on inhibition of ^{3}H-deoxy-glucose uptake in H4IIE cells. The data are presented as means ± SE for three independent experiments. There were no significant differences between groups as determined by ANOVA.

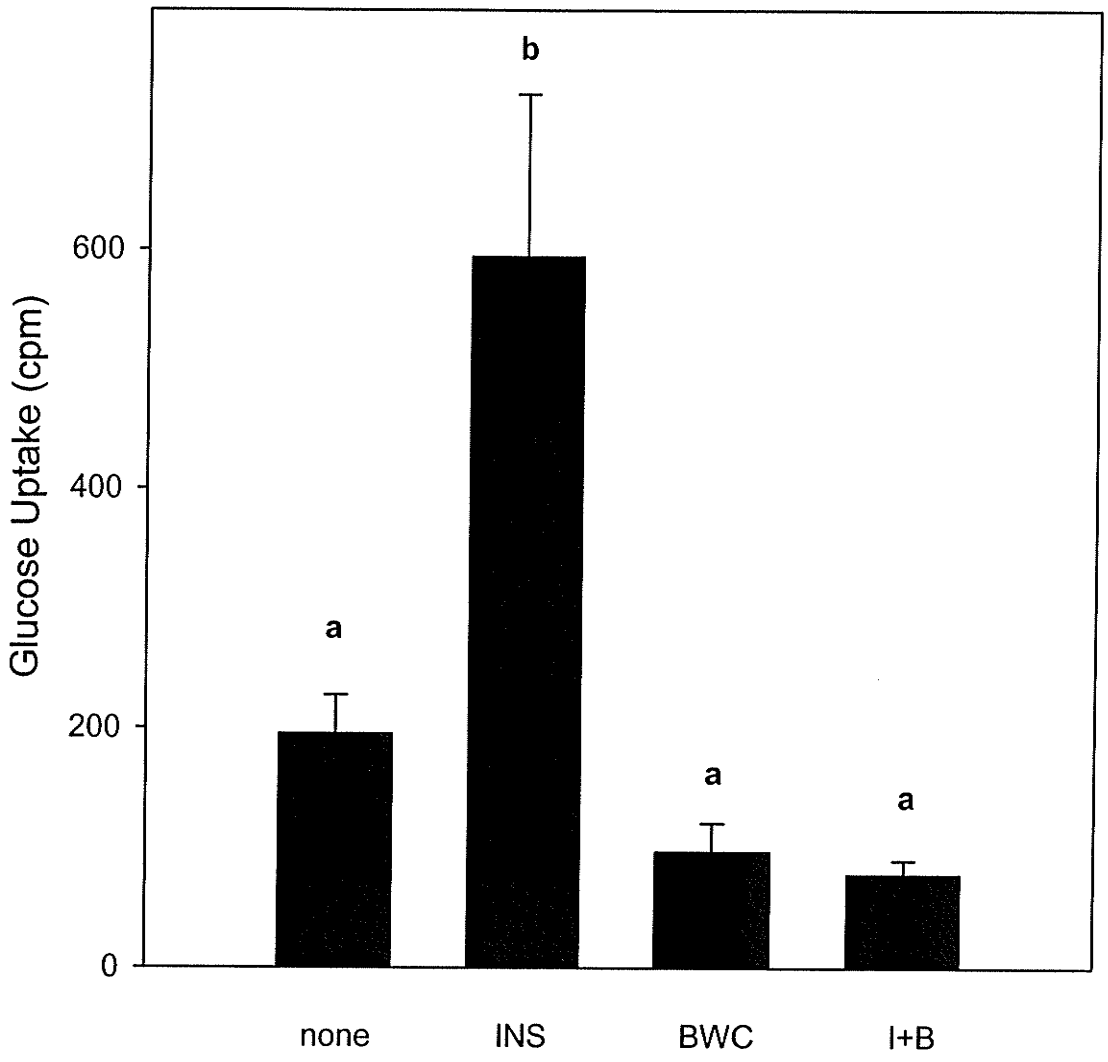

Figure 8-3. The effects of the buckwheat concentrate (BWC) and insulin (INS) on basal ^{3}H-deoxy-glucose uptake in H4IIE cells. The effects of the BWC on INS-stimulated (I+B) glucose uptake were also determined. The data are presented as means ± SE for three independent experiments. Statistical significance ($p<0.05$) was determined by ANOVA and Duncan's multiple range test and bars with different letters are significantly different.

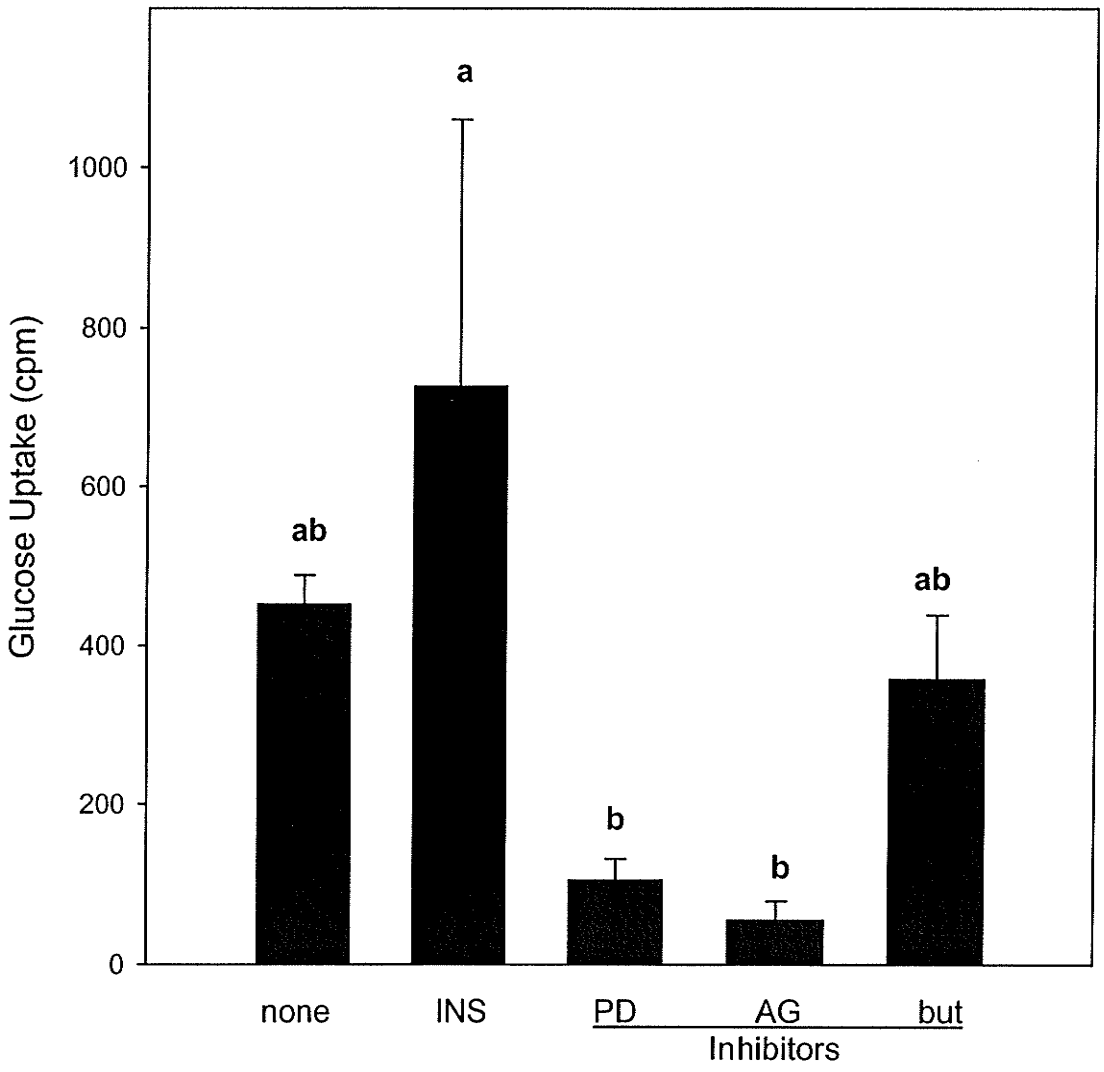

Figure 8-4. The effects of inhibiting the phosphorylation of MAPK by PD98059 (PD), insulin receptor tyrosine kinase by AG1024 (AG) and phospholipase D by butanol (but) on insulin (INS)-stimulated ^{3}H-deoxy-glucose uptake in H4IIE cells. The data are presented as means ± SE for three independent experiments. Statistical significance ($p<0.05$) was determined by ANOVA and Duncan's multiple range test and bars with different letters are significantly different.

8.5 Discussion

The main finding of this study was that the BWC inhibited glucose uptake in H4IIE cells whereas D-CI and MI stimulated glucose uptake similar to insulin. Insulin lowers blood glucose in part by increasing hepatic glucose utilization and storage and thus stimulating glucose uptake in the liver. We previously reported that the BWC reduced hyperglycemia in STZ rats (Kawa *et al*, 2003; Chapter 6). The BWC contains D-CI and MI, insulin-mimetic compounds. In the present study, we hypothesized that the BWC, as a source of these compounds, would increase glucose uptake in H4IIE cells as a result of increased enzyme activity related to glucose utilization and/or storage. However, D-CI and MI were effective for stimulating glucose uptake whereas the BWC was not.

The increase in glucose uptake by H4IIE hepatoma cells represents an increase in glucose utilization rather than a change in glucose transporter activity as insulin-stimulated glucose uptake in the liver is due to increased metabolic activity. For example, insulin increases expression of hepatic glucokinase, an enzyme that converts glucose to a form that is both trapped in the cell and capable of being further metabolized (Shimomura *et al*, 2000; King, 2002). In the present study we demonstrated that insulin-stimulated glucose uptake requires stimulation of cell signal transduction proteins leading to downstream metabolic activities such as glycogen synthesis and gene transcription.

The effects of D-CI and MI on glucose uptake in H4IIE cells were similar to that of insulin in the present study. This finding is in agreement with previous studies reporting insulin-mimetic effects of IPGs containing these compounds

(Field, 1997; Jones and Varela-Nieto, 1998, 1999). The glucose-lowering effects of free D-CI and MI in animal models of DM may also explain our results showing these compounds effectively increase glucose uptake. In contrast, results from this study suggest that the BWC does not stimulate glucose utilization in the liver and thus does not increase the rate of glucose influx into these cells. This suggests that the presence of D-CI and MI in the BWC may not be responsible for the previously observed antihyperglycemic effects of the BWC (Kawa *et al*, 2003). Other compounds present in the BWC may be responsible for the glucose-lowering effects we reported in STZ rats. Buckwheat also contains fagopyritols, flavonoids, and lignans, thus one of these components may have contributed to the antihyperglycemic effects of the BWC (Horbowicz and Obendorf, 1994; Watanabe, 1998; Quettier-Deleu et al, 2000; Holasova, 2002; Yokozawa, 2002; Fabjan, 2003; Setchell, 1995).

It is also possible that the BWC is acting via a different mechanism than increasing hepatic glucose uptake to reduce hyperglycemia. Glucose transport is also achieved via Na^+ dependent glucose transporters (SGLTs), which are mostly known for their mediation of intestinal absorption and renal reabsorption of glucose (Wood and Trayhurn, 2003). Inhibitors of SGLT have been reported to be antidiabetic agents, by potentially increasing urinary glucose excretion and/or decreasing intestinal glucose absorption (Oku *et al*, 1999). Another potential mechanism is that the BWC may increase GLUT4 translocation, thus stimulating skeletal muscle and adipose tissue glucose uptake which has nothing to do with the effects noted with H4IIE cells. It is also possible that the previously

observed antihyperglycemic effects of the BWC are related to plasma glucagon-like peptide-1 (GLP-1) and/or glucose-dependent insulinotropic polypeptide (GIP), which are released from the small intestine upon nutrient absorption, and stimulate insulin secretion from pancreatic beta cells (Enc *et al*, 2001). The effects of the BWC on these insulin-dependent and independent mechanisms for reducing blood glucose concentrations can be evaluated in future studies.

The reason for inhibition of basal and insulin-stimulated glucose uptake by the BWC in the H4IIE cells can also be addressed in future studies. Perhaps this effect of the BWC in hepatocytes is somehow related to its antihyperglycemic actions elsewhere in the body. Further investigations may reveal the significance and meaning of these results.

9. Discussion

The main findings of this research were that 1) urinary excretion of D-CI and MI was elevated in animal models of DM-1 and DM-2, concomitant with hyperglycemia and glucosuria, 2) urinary excretion of D-CI and MI was elevated in individuals with DM-1, and urinary D-CI and MI were associated with hyperglycemia and glucosuria in humans, 3) administration of a BWC to normal rats given a glucose load and STZ diabetic rats resulted in lowered blood glucose concentrations, 4) in H4IIE cells, a BWC stimulated phosphorylation of MAPK and $p70^{S6K}$ (Thr^{421}), insulin-stimulated cell signal transduction proteins, and 5) a BWC inhibited insulin-dependent glucose uptake by H4IIE cells.

The pattern of urinary inositol excretion in DM is related to the altered bioactivity and metabolism of D-CI and MI, components of insulin-mimetic compounds, that exists in DM. Previous reports on the urinary excretion of these components have disagreed on the pattern of inositol excretion that exists in DM. Clarification of this pattern may provide a biomarker for altered inositol metabolism in DM and an outcome measurement for further investigations of the contribution and role of inositol metabolism in insulin signaling and pathogenesis of DM. Therefore, objectives of the present research were to determine the pattern of inositol excretion that exists in DM animal models and individuals with DM. In STZ rats and db/db mice (DM-1 and DM-2 animal models, respectively), we found that urinary D-CI and MI were elevated. In contrast, urinary inositol excretion in fa/fa Zucker rats, a model of IGT, was normal. Our investigation of the pattern of urinary inositol excretion in humans with and without DM revealed

that individuals with DM-1 also have elevated urinary D-CI and MI. In DM-2 subjects, however, urinary MI and D-CI excretion was not different from DM-2 matched controls or DM-1 controls. Our DM-2 participants, however, did not have hyperglycemia or glucosuria and were no different from either control group for these indices of diabetes.

Thus, our findings indicate elevated inositol excretion in DM appears to be related to hyperglycemia and accompanying glucosuria. In animals and humans without glucosuria (fa/fa Zucker rats and DM-2 participants), urinary inositol excretion was not elevated whereas in STZ rats, db/db mice, and individuals with DM-1, who all had chronic hyperglycemia and glucosuria, urinary excretion of inositols was significantly elevated compared to their non-diabetic counterparts. One possible reason for these observations could be that elevated blood glucose resulting in decreased glucose reabsorption in the kidney promotes increased conversion of glucose to MI and the products of its metabolism (D-CI and glucuronic acid). Another possibility is that decreased incorporation of MI and/or D-CI into IPGs leads to elevated excretion of these compounds. Elevated D-CI could also be the result of elevated MI excretion, as MI is converted to D-CI.

In the present research, the pattern of urinary inositol excretion was determined in three different animal models of DM as well as in humans with DM-1 and DM-2. Previous studies have mainly focused on the pattern of inositol excretion in humans and there is little research in this area that involves animal models of DM (Suzuki *et al*, 1991, 1994; Kennington *et al*, 1990; Ostlund *et al*, 1993). Furthermore, different methods for urinary inositol analysis have been

used in the past and thus make it difficult to compare results across studies. An advantage to this research is that the analysis included both humans and animal models with DM-1 and DM-2, and IGT, and was performed using the same method in the same laboratory. The group of individuals with self-declared DM-2 (managed by diet only) were not different from their matched controls for hyperglycemia and glucosuria, suggesting they had well-controlled DM. This may have contributed to the lack of differences in urinary inositol excretion between the DM-2 group and the controls. Thus, the absence of a poorly controlled DM-2 group as part of our analysis is a limitation of this work.

Overall, results from this research indicate that urinary inositol excretion is elevated in DM, concomitant with hyperglycemia and glucosuria. Further research can clarify the pattern of inositol excretion in DM-2 versus IGT (early stages of the disease) and can also evaluate the factors in DM that contribute to this urinary excretion pattern (altered IPG content or hyperglycemia and glucosuria).

Administration of free D-CI has demonstrated antihyperglycemic effects in DM most likely due to a DM-dependent alteration in the metabolism and bioactivity of insulin-mimetic IPGs that contain D-CI (Ortmeyer *et al*, 1993; Fonteles *et al*, 2000). Buckwheat contains D-CI and galactosyl derivates of D-CI known as fagopyritols and is a potential source of these compounds in treatment of DM. In addition, buckwheat contains MI, which has also been identified as part of an insulin-mimetic IPG compound. Thus, in the present research, we investigated the effects of a buckwheat concentrate (BWC) on lowering blood

glucose concentrations in a diabetic animal model. We found that the BWC effectively lowered blood glucose concentrations in normal rats given glucose and STZ diabetic rats. Although the BWC provided a concentrated source of these compounds (D-CI, MI, and fagopyritols), it was not purified and therefore other components present in the extract may have contributed to the observed effects. However, since our results were similar to those reported in previous studies using free D-CI, we concluded that the observed effects were due to this compound.

In an attempt to determine the mechanism for the observed antihyperglycemic effects of the BWC, we investigated the insulin-mimetic activities of the BWC with H4IIE cells. In this cell type, the BWC stimulated phosphorylation of MAPK and $p70^{S6K}$ (Thr^{421}), effects that are also observed following binding of insulin to its receptor in target tissues. MAPK is a signal transduction protein whose phosphorylation results in the downstream regulation of gene transcription. The cellular protein $p70^{S6K}$ (Thr^{421}) is also stimulated by insulin and its phosphorylation is required for cell growth. Downstream from $p70^{S6K}$ is the ribosomal protein S6 which is necessary for protein synthesis. Although insulin can effectively stimulate $p70^{S6K}$ phosphorylation of both the Thr^{421} and Thr^{389} residues, a prerequisite for S6 phosphorylation, the BWC was only able to stimulate phosphorylation of the Thr^{421} residue. The role of MAPK and $p70^{S6K}$ (Thr^{421}) activation in relation to the glucose lowering effects of the BWC *in vivo* is not known, but may be related to altered expression or activation of enzymes involved in glucose metabolism or proteins that were not determined

in the present research. Further research to investigate the significance of these findings in relation to glucose metabolism is warranted.

The observed effects of the BWC on MAPK and $p70^{S6K}$ (Thr^{421}) required the participation of PLD and a member of the src kinase family, but did not require stimulation of insulin receptor phosphorylation. This suggests that the BWC is acting by a mechanism independent of insulin receptor activation for stimulating phosphorylation of these proteins. Furthermore, the cell signaling pathways activated by the BWC are independent from other insulin-stimulated pathways including those of PI3K/Akt. The entry point for the BWC within these signaling pathways was not determined in the present research, however, future work may provide further understanding in this area.

BWC-dependent stimulation of glucose uptake in H4IIE cells was also investigated as part of this reseach. Interestingly, the BWC inhibited basal glucose uptake and insulin-stimulated glucose uptake in these cells. The reason for this inhibition was not addressed in the present work, however, further investigations may reveal the significance and meaning of these results. The lack of glucose uptake stimulation in H4IIE cells by the BWC suggests that the BWC is acting via a different mechanism than increasing hepatic glucose uptake to reduce hyperglycemia. Other possibilities for the glucose lowering mechanism of the BWC include activation of GLUT4 translocation in skeletal muscle and adipose, or mechanisms involving SGLTs, GLP-1 and/or GIP.

We also compared the effects of chemically synthesized D-CI and MI standards on cell signal protein activation and glucose uptake to determine the

contribution of these compounds to the observed effects of the BWC. In contrast to our observation that the BWC was effective for stimulation of MAPK and $p70^{S6K}$ (Thr^{421}), D-CI and MI did not activate these proteins. Thus, we concluded that D-CI and MI did not contribute to the BWC-stimulation of MAPK and $p70^{S6K}$ (Thr^{421}) and perhaps other compounds in the BWC were responsible for the observed effects.

Interestingly, D-CI effectively increased glucose uptake in H4IIE cells, comparable to insulin. A similar observation was made with MI, although the results were not significant. This is in contrast to the BWC, which inhibited glucose uptake in these cells, indicating it is not an effective source of these compounds for stimulating glucose uptake. We concluded that free D-CI and MI do have insulin-mimetic effects on stimulation of glucose uptake in H4IIE cells, although they did not activate any insulin-stimulated signal transduction proteins evaluated in the present research. This suggests that different pathways lead to glucose uptake in H4IIE cells besides those involving the signal transduction proteins we measured.

The present research investigated the antihyperglycemic effects of the BWC and its mechanism of action. We were able to show that a BWC produced glucose-lowering effects in a diabetic animal model. Previous studies reporting antidiabetic properties of buckwheat have used whole buckwheat rather than a concentrated extract (Lu *et al*, 1992; Wang *et al*, 1992; Bijlani *et al*, 1985), making identification of bioactive components more difficult. Furthermore, a concentrated extract has the potential to be used not only as a nutraceutical but

also as a functional food ingredient. Previous studies also lacked detailed study descriptions (Lu *et al*, 1992; Wang *et al*, 1992; Bijlani *et al*, 1985), thus, our findings on the antidiabetic effects of buckwheat in well-controlled conditions provide confirmation to earlier reports. We reported the acute effects of the BWC in STZ rats, however, the effects of chronic supplementation with buckwheat in this animal model was not addressed in this research. Using H4IIE cells, we investigated the mechanism of action for the glucose-lowering effects of the BWC. We chose this cell type because the liver plays a central role in glucose metabolism, and insulin is able to stimulate glucose metabolic pathways in these cells. However, the effect of the BWC on glucose uptake and metabolism in other tissues was not evaluated. Components in buckwheat that may have contributed to the observed effects in the present research include protein or peptides, fibre, resistant starch, and flavonoids for the glucose-lowering effects in STZ rats, whereas peptides, flavonoids, or other phytochemicals are more likely candidates for the observed effects of the BWC in H4IIE cells. The presence of these compounds in the BWC was not specifically identified in this research, however, future work can determine their potential contribution to the observed antihyperglycemic and insulin-mimetic effects of the BWC.

Thus, results from this research suggest that 1) urinary excretion of D-CI and MI is elevated in diabetes, concomitant with hyperglycemia and glucosuria and 2) a BWC has insulin-mimetic effects in STZ rats, where it effectively lowered serum glucose concentrations, and in H4IIE cells, where it demonstrated activation of insulin-stimulated cell signal transduction proteins. Future work can

address 1) the relationship between elevated inositol excretion in diabetes and the pathogenesis of the disease, 2) the significance of MAPK and $p70^{S6K}$ (Thr^{421}) phosphorylation by the BWC in relation to glucose metabolism, as well as 3) the reason for inhibition of glucose uptake by the BWC in H4IIE cells.

Overall, the approach for this thesis is unique in that it combines analytical chemistry, *in vivo* response and mechanistic cell culture studies for functional food/nutraceutical research. The findings of this research suggest that buckwheat has antidiabetic effects and may be useful for the management of diabetes. This can have implications for the buckwheat industry in Canada. Also, the pattern of urinary inositol excretion reported here can contribute to current knowledge of the pathogenesis of DM, and may be useful as a biomarker or lead to novel treatments for the disease.

10. References

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11. Appendices

Appendix A: Method details

Appendix B: Supplementary tables for manuscripts

Appendix C: Additional experiments evaluating the effects of buckwheat supplementation in animal models

Appendix D: Additional experiments evaluating the molecular and cellular mechanisms for the antihyperglycemic effects of buckwheat

Appendix A: Method details

Serum and urine glucose

The procedure to measure serum and urine glucose concentration is based on 2 coupled enzymatic reactions using glucose oxidase (Reaction #1) and peroxidase enzyme (Reaction #2).

Reaction #1: Glucose + H_2O + O_2 $\rightarrow$ Gluconic Acid + H_2O_2

Reaction #2: H_2O_2 + o-Dianisidine (colorless) $\rightarrow$ Oxidized o-Dianisidine (brown color)

The intensity of the brown color measured at 425-475 nm is proportional to the original glucose concentration in the serum and urine samples.

a) Reagents

- Glucose Assay Kit (Sigma, Procedure #510-A)
    - Peroxidase Glucose Oxidase (PGO) Enzymes (Product #510-6)
    - Enzyme solution (Prepared by adding contents of 1 capsule of PGO enzymes to 100 mL distilled water in an amber bottle)
    - o-Dianisidine Dihydrochloride (Product #510-50)
    - Color Reagent Solution (Prepared by reconstitution one vial of o-Dianisidine Dihydrochloride with 20 mL water)
    - Combined Enzyme-Color Reagent Solution (Prepared by combining 100 mL enzyme solution and 1.6 mL Color Reagent Solution)
    - Glucose Standard Solution (Product #635-100, 100 mg/dL (5.56 mmol/L) in 0.1% benzoic acid solution)

b) Procedure

Glucose standard was diluted and used to establish the standard curve (0-25 mg/dL). Urine and serum samples were diluted with deionized water to determine concentration based on the standard curve. Urine and serum samples were diluted 800-fold and 70-fold, respectively, for db/db mice. Serum samples were diluted 10-fold for C57BL/6 mice, 20-fold for Sprague-Dawley rats, and 30-fold for fa/fa and lean Zucker rats whereas urine samples for these animals were undiluted. Urine samples for STZ rats were diluted 500-fold. Serum samples for glucose tolerance tests were diluted 20- and 30-fold for STZ rats, 10- and 20-fold for fa/fa and lean Zucker rats, respectively. Twenty μL of samples, standards and blanks were pipetted in triplicate into 96-well Polystyrene Microtiter Plates (Corning Glass Works). Two-hundred μL combined enzyme-color reagent solution was pipetted into the wells using a multi-channel pipette. The plate was gently mixed, covered with foil and incubated at room temperature (18-26°C) for 45 minutes. Absorbencies were read at 450 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation). Concentration of glucose in the samples was calculated based on the standard curve.

c) Calculation

$$\text{Glucose concentration (mg/dL)} = \frac{\text{Concentration of Standard} \times [A]\text{Sample}}{[A]\text{ Standard}} \times \text{Dilution}$$

Where [A] = absorbance

Conversion of units: mmol/L = mg/dL x 0.0555

Serum insulin

Insulin in the serum of mice and rats was assayed using a sensitive rat insulin radioimmunoassay kit (#SRI-13K, Linco Research Inc., St Charles, MO). The procedure follows the basic principle of radioimmunoassay where there is a competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The amount of ^{125}I-insulin bound is inversely proportional to the concentration of unlabelled insulin.

a) Reagents

- Assay Buffer: 0.05 M Phosphosaline (pH 7.4) containing: 0.025 M EDTA, 0.08% Sodium Azide, 1% RIA grade Bovine Serum Albumin
- Sensitive Rat Insulin Antibody
- ^{125}I-Insulin label (<3 μCi/27 mL) hydrated in assay buffer containing Normal Guinea Pig IgG as carrier
- Insulin standards (0.02 – 1.0 ng/mL) and Quality controls
- Precipitating reagent

b) Procedure

On day 1, serum insulin samples were diluted in Assay Buffer (25-fold for db/db serum, 10-fold for C57BL/6, Sprague-Dawley, lean Zucker serum, and 100-fold for fa/fa Zucker serum) to be within range of the standard curve. Assay buffer was pipetted into Borosilicate glass (12 x 75 mm) tubes: 300 μL into 2 non-specific binding tubes, 200 μL into 2 total binding reference tubes, and 100 μL into tubes for standards, controls, and unknowns. One hundred μL of standards, quality controls, or diluted samples were pipetted in duplicate into

appropriate tubes. One hundred μL of rat insulin antibody was pipetted into all tubes except total count and non-specific binding tubes. Tubes were vortexed, covered with parafilm, stored in a metal test tube rack in a sealed moist plastic container, and incubated overnight at 4°C. The following day, 100 μL ^{125}I-insulin was pipetted into all tubes. Tubes were vortexed, covered with parafilm, stored in a metal test tube rack in a sealed moist plastic container, and incubated overnight at 4°C. On the third day, 1.0 mL precipitating reagent was added to all tubes except total count tubes. Tubes with the precipitating reagent were vortexed, incubated for 20 minutes at 4°C, and centrifuged for 40 minutes at approximately 2000 x g to achieve a firm pellet. The supernatant fraction was decanted and tubes were held inverted for 10 seconds for complete blotting of all liquid. Remaining pellets were counted for ^{125}I in a gamma counter (Beckman Gamma 8000, Scientific Instruments).

c) Calculation

- Total count tubes = tubes with ^{125}I only
- Non-specific binding (NSB) tubes = tubes with ^{125}I and precipitating agent (no insulin antibody, sample or standard)
- Total binding reference tubes = tubes with all reagents except sample or standard
- Duplicate counts for Total count tubes, NSB tubes, Total Binding tubes (reference, Bo), and all duplicate tubes for standards and samples were averaged.

- Non-specific binding count was subtracted from each average count (except for Total count).

$$\text{Percentage of maximum binding (\%B/Bo)} = \frac{\text{Sample or Standard cpm}}{\text{Total Binding Reference cpm}} \times 100$$

A reference curve (based on the standards) was developed using the Prism computer software program to calculate the equation of the line and to solve for unknowns. Insulin concentrations (ng/mL) for unknown samples and quality controls were then determined by interpolation on the standard reference curve and multiplied by the appropriate dilution factor.

Urine creatinine

Creatinine is a urinary excretion product that is proportional to body muscle mass (Bowers and Wong, 1980). Since urinary creatinine excretion for the body is relatively constant, it was used as a basis for expressing urinary concentration of glucose and inositols in this research. The corrected urinary values thus take into account differences in urine volume as reflected by creatinine excretion. A colorimetric creatinine assay kit (Sigma Diagnostics, Procedure #555) was adapted to a microplate method and was used to determine the creatinine concentration in urine collected from db/db mice, STZ rats, fa/fa Zucker rats and their control counterparts. This procedure employs the principle that upon contact with alkaline picrate solution, a sample will form an orange/yellow colour. This colour is derived solely from creatinine and is destroyed by the addition of an acidic pH solution. Therefore, the difference in colour intensity, measured at 500 nm before and after acidification, is proportional to creatinine concentration.

a) Reagents

- Creatinine assay kit (Sigma, Procedure #555)
- Creatinine Color Reagent (Product #555-1, 0.6% Picric Acid, sodium borate and surfactant)
- Acid Reagent (Product #555-2, mixture of sulfuric acid and acetic acid)
- Sodium Hydroxide Solution (Product # 930-55, 1.0 N)
- Alkaline Picrate Solution (Prepared by mixing 5 volumes of Creatinine Color Reagent with 1 volume of Sodium Hydroxide Solution)

- Creatinine Standard (Product #925-3, Creatinine, 1.0 mg/dL (μmol/L), 3.0 mg/dL (265 μmol/L), 10 mg/dL (μmol/L), and 15 mg/dL (1325 μmol/L) in 0.02 N HCl)

b) Procedure

Urine samples from mice and rats were diluted 20-fold using distilled water. Creatinine standards were used to develop a standard curve (0-15 mg/dL from which unknown concentrations were determined. The alkaline picrate reagent was prepared fresh daily and stored in the dark at room temperature.

Samples, standards and buffer blanks of 20 μL were pipetted in triplicate into a 96-well Polystyrene Microtiter Plate (#25880-96, Corning Glass Works, Corning, NY). Two-hundred μL alkaline picrate reagent was pipetted into the wells using a multi-channel pipette. The plate was placed in the drawer of a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, CA), mixed and incubated for 10 minutes at room temperature before reading the initial absorbance at 500 nm. The plate was removed from the drawer, 7 μL acid reagent was pipetted into the wells using a multi-channel pipette and the plate was mixed in the spectrophotometer. The acid reagent was allowed to incubate for 5 minutes and then a second reading was recorded. Absorbencies were read at 500 nm and sample concentrations were determined based on the standard curve.

c) Calculation

$$\text{Creatinine Concentration (mg/dL)} = \frac{\text{Concentration of Standard} \times [A]\ \text{Sample}}{[A]\ \text{Standard}} \times \text{Dilution}$$

Where [A] = absorbance

Buckwheat milling

D-*chiro*-inositol (D-CI), *myo*-inositol (MI), and fagopyritols are concentrated in the testa and embryo of the buckwheat groat however these seed parts comprise only 25% of the buckwheat groat whereas the majority of the groat is the endosperm portion (Li and Zhang, 2001). A milling procedure was developed to isolate a fraction from buckwheat containing the cotyledon, embryo, and testa with a minimal amount of endosperm. To further concentrate these components, the milled fraction was subjected to selective solvent extraction and evaporation as described in Chapter 6 (Section 6.3). The resulting liquid concentrate was used in experiments involving buckwheat supplementation in animals in order to limit the amounts of whole buckwheat and milled buckwheat needed to achieve high doses of these compounds through dietary consumption.

For the milling procedure, moisture concentration was increased from 12 to 17% in dehulled buckwheat seeds 2 hours prior to milling by the addition of water to buckwheat. Moisture was then increased by an additional 1% just prior to milling. Seeds were milled using a Buhler mill (MLU-202) according to the manufacturer's specifications. The grinding gaps were set as follows:

B2 = 0.12 mm

B3 = 0.10 mm

1M = 0.07 mm

3M = 0.03 mm

The screen sizes for the breaks and reductions are provided in the Table A-1.

Table A-1. Screen Sizes

Grinding Passages		Top Screen		Bottom Screen	
		W / XX	microns	XX	microns
First Break	B1	40 w	420	10 xx	132
Second Break	B2	40 w	420	10 xx	132
Third Break	B3	45 w	360	10 xx	132
First Reduction	1M	9 xx	153	9 xx	153
Second Reduction	2M	9 xx	153	9 xx	153
Third Reduction	3M	9 xx	153	9 xx	153

Seeds were passed through a series of corrugated rolls or break rolls followed by sifting to separate the coarse, flexible particles (testa, cotyledon and embryo) from the fine, dry particles (endosperm). The material passing over the first break sifter top screen was sent to the second break for further grinding and likewise from second to third break. The material passing over the third break sifter top screen was collected as bran. The material passing over the bottom screen of the first, second and third break sifter were collected together and was sent to the first reduction roll for further grinding. The material passing through the bottom screen of the first, second and third break sifter was collected as flour consisting of dry, fine particles of endosperm. The overs of the first reduction sifter screen was sent to the second reduction sifter and likewise from second to third reduction sifter. The overs of the third reduction sifter screen was collected

as shorts, which were more fine particles of testa, cotyledon and embryo with some remaining endosperm attached. The throughs of the first, second and third reduction sifter screens were collected as flour or white fraction of finely ground endosperm particles. This shown in the Figure A-1.

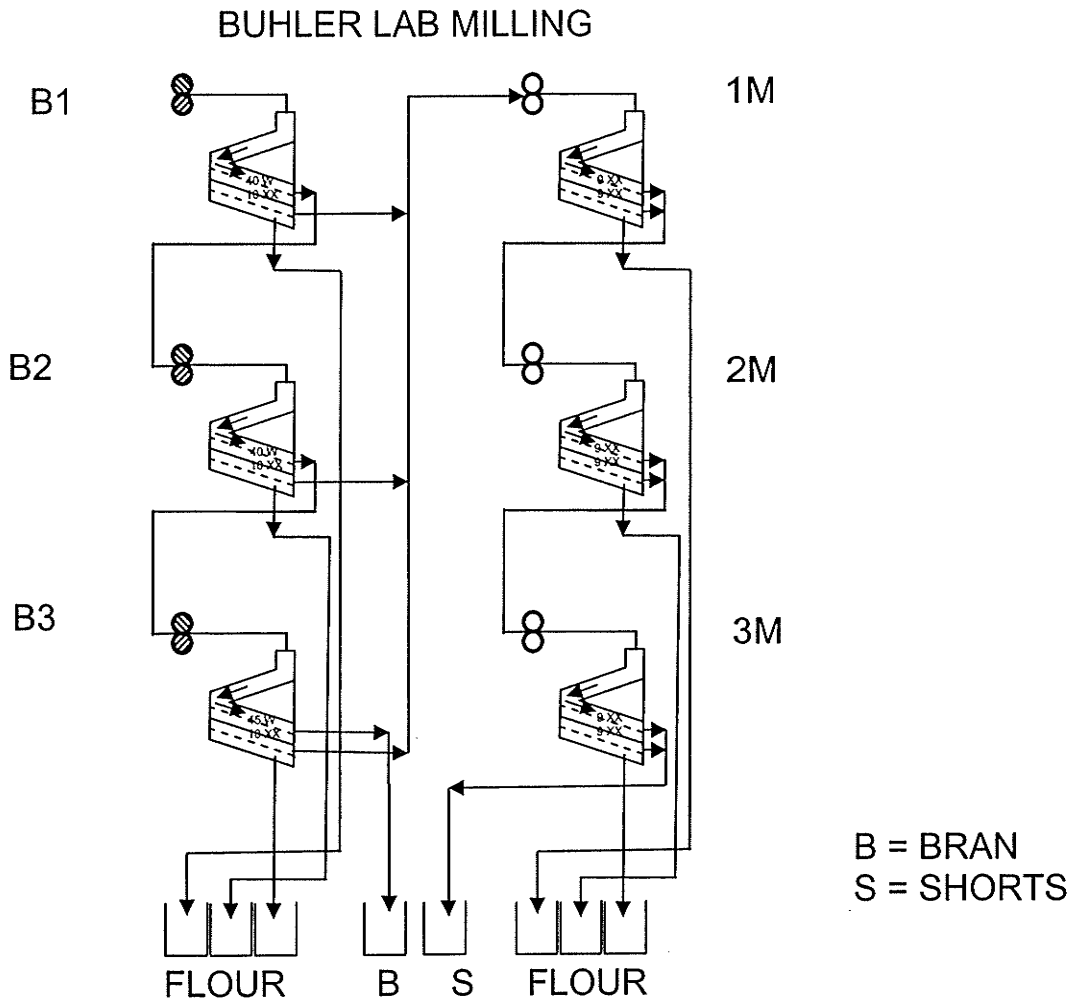


Figure 1. Buhler Lab Milling Flowsheet for Buckwheat

Soluble carbohydrates, including D-CI, MI and fagopyritols, were extracted from the combined bran and shorts fractions as described in Chapter 6 (Section

6.3) and used for buckwheat supplementation in animal studies (unless otherwise indicated) and in the treatment of H4IIE cells.

Isolation of protein from H4IIE cells

Preparation of protein samples involves the use of formulated extraction buffers containing specific concentrations and classes of detergents to disrupt cells for the harvest of cytoplasmic, membrane-associated, cytoskeletal and nuclear proteins.

a) Reagents

- Smooth muscle cell phosphate-buffered saline (SMC-PBS):

  0.9% NaCl, 0.1 M Na_3PO_4 (pH 7.1)

- 2 x sodium dodecyl sulfate (SDS)/gel loading buffer:

  0.125 M Tris•HCl (pH 6.8), 2% SDS, 10% glycerol

b) Procedure

Following incubation with treatments, media was removed from each well using a Pasteur pipette and vacuum suction. Cells were then rinsed with approximately 1 mL of SMC-PBS and the SMC-PBS was removed using the same Pasteur pipette and vacuum suction system. Cellular protein extracts were prepared by addition of 200 µL 2 x SDS/gel loading buffer to cells in 12-well culture dishes and incubated at room temperature on a shaker for 5 minutes. The samples were transferred to 1.5 mL microfuge tubes, sonicated for 5 seconds at 60 Hz, and stored at -20°C.

Determination of protein concentration in cellular extracts

The concentration of protein in cellular extracts was assayed using the BCA protein assay reagent kit (Pierce). The procedure to measure protein concentration is based on two reactions. It combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the colorimetric detection of the cuprous cation (Cu^{+1}) using a reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion, and exhibits a strong absorbance at 562 nm.

Reaction 1: Protein (peptide bonds) + Cu^{+2} $\xrightarrow{OH^-}$ tetradentate-Cu^{+1} complex

Reaction 2: Cu^{+1} + 2 Bicinchoninic Acid (BCA) $\longrightarrow$ BCA- Cu^{+1} ternary complex (purple colored)

The intensity of the purple color measured at 562 nm is proportional to the original protein concentration in the cellular extracts.

a) Reagents

- BCA Protein Assay Reagent Kit (Pierce, Kit #23225)
  - BCA Protein Reagent A (Product # 23223)
  - BCA Protein Reagent B (Product # 23224)
  - Albumin Standard (Product # 23209)

b) Procedure

Protein standards, prepared by diluting the 2.0 mg/mL Albumin Standard (BSA stock standard) in 2 X SDS/gel loading buffer, were used to establish the

standard curve (0-2 mg/mL). Ten μL of standards and samples were pipetted in triplicate into 96-well plates (Nunc). The working reagent was prepared by adding 50 parts of BCA Reagent A with 1 part of BCA Reagent B. Two hundred μL of the working reagent was added to each well using a multi-channel pipette. The plate was sealed with parafilm, covered and incubated at 37°C for 30 minutes. Absorbencies were read at 550 nm using a microplate reader (Molecular Devices Thermo max microplate reader). The concentration of protein in the samples was calculated based on the standard curve.

c) Calculation

$$\text{Protein Concentration } (\mu g/\mu L) = \frac{\text{Concentration of Standard x [A] Sample}}{\text{[A] Standard}}$$

Where [A] = absorbance

Preparation of polyacrylamide gels

Polyacrylamide gels containing SDS provide a denaturing media in which proteins may be separated by molecular mass. By altering the concentration of acrylamide monomers, gel porosity can be varied over a wide range to meet specific separation requirements.

a) Reagents

- 20% (w/v) acrylamide (Roche)
- 20% (w/v) sodium dodecyl sulfate (SDS) (Invitrogen)
- 1.5 M Tris•HCl Buffer (pH 8.8)
- 0.5 M Tris•HCl Buffer (pH 6.8)
- H_2O-saturated butanol
- 10% (w/v) ammonium persulfate (APS) (BioRad)
- N, N, N', N' – Tetramethylethylenediamine (TEMED) (Sigma)
- General laboratory chemicals were obtained from Fisher or Sigma

b) Procedure

Depending on the desired gel thickness, 0.75 or 1.0 mm spacers were placed between a large and small glass plate. Glass plates were inserted into the sandwich clamp assembly and placed into the casting stand. After spacers and glass plates were properly positioned, clamp screws were tightened and the sandwich clamp assembly was attached into the casting slot of the stand. A separating gel solution was prepared (7.5% acrylamide, 0.34 M Tris•HCl (pH 8.8), 0.1% SDS, 0.1% APS, 8 x 10^{-4} % TEMED) and poured between the glass plates with a Pasteur pipette. Approximately 200 μL of H_2O-saturated butanol

was gently layered onto the top of the separating gel solution. The gel was left to polymerize for approximately 30 minutes. The butanol was poured off and the gel was rinsed with deionized water. Excess water was removed and the appropriate well comb was inserted between the plates. A stacking gel was prepared containing 5% acrylamide, 0.13 M Tris•HCl (pH 6.8), 0.1% SDS, 0.1% APS, and 0.25% TEMED. The stacking gel was added to the top of the separating gel and left to sit until completely polymerized, which required approximately 10 minutes. The comb was removed, the gel was rinsed with deionized water and excess water was removed.

SDS-PAGE

Electrophoresis uses an electrical field to separate proteins and nucleic acids by drawing the molecules through a porous gel matrix. Polyacrylamide gel electrophoresis, containing SDS, provides a denaturing media in which proteins may be separated by molecular mass.

a) Reagents

- H_2O-saturated bromophenol blue (Fisher)
- 2-mercaptoethanol (Sigma)
- SDS-PAGE Electrode Buffer:

  0.125 M Tris, 0.96 M Glycine, 0.5% SDS
- Bromophenol blue (Fisher)

b) Procedure

Aliquots of cell homogenates containing equal amounts of protein (5 – 10 μg) were mixed with bromophenol blue (0.5% (w/v) final concentration) and 2-mercaptoethanol (5% (v/v) final concentration) and heated at 95°C for 5 minutes. Sandwich clamp assemblies were attached to the inner-cooling core and placed into the lower buffer chamber of the electrophoretic apparatus. SDS-PAGE electrode buffer was added to the interior of the inner-cooling core. Molecular mass markers and protein samples were loaded onto gels. The surrounding area of the casting box was filled with SDS-PAGE electrode buffer. The lid was attached to the lower buffer chamber and connected to a power supply of 20 mA of current per gel. Gels were run until the dye-front reached the bottom of the glass plates (approximately 1 hour).

Gel transfer

SDS-PAGE is used to separate protein samples in relation to size. Once this process is complete, the proteins in the gel can be electrophoretically transferred onto a polyvinylidenedifluoride (PVDF) membrane for Western immunoblotting.

a) Reagents

- 5 x Transfer buffer:

  0.16 M Tris, 0.81 M Glycine

b) Procedure

1 x Transfer buffer (20% methanol, 25 mM Tris, 130 mM glycine), prepared by dilution of 5 x Transfer buffer and addition of methanol, was placed into a large container. The PVDF membrane (Millipore) was moistened in a small amount of methanol and then equilibrated in the prepared 1 x Transfer buffer for at least 5 minutes. The glass plates containing the gel were removed from the sandwich clamp assembly. One glass plate was removed and the stacking gel was discarded. The separating gel was then pressed onto gel blotting paper (Schleicher and Schuell, Mandel) and the second glass plate removed. The PVDF membrane was taken from the buffer solution and placed on top of the gel. With all components submerged in the buffer solution, a fiber pad was placed on top of the negative side of an open transfer cassette. The filter paper with attached gel and membrane was added with membrane facing upwards. A second filter paper and fiber pad were added and any trapped air bubbles were removed. The cassette was closed and placed into the electrode module with the negative side facing the appropriate negative side of the module. A magnetic

stir bar and cooling unit were added to the buffer tank and the buffer solution was added to the tank. The apparatus was placed onto a magnetic stirrer and the proteins were transferred to PVDF membrane at 100 V for 1 hour in the buffer solution.

Western immunoblotting

Western blotting is a technique used to visualize protein(s) located on PVDF membranes. Incubating the membrane with the appropriate primary antibody allows the protein to be tagged for detection with a secondary antibody conjugated to an enzyme. This enzyme catalyzes the production of a luminescent compound detectable upon exposure to autoradiographic film.

a) Reagents

- 5 x Tris-buffered saline with Tween (TBST):

  0.1 M Tris•HCl (pH 7.4), 0.75 M NaCl, 0.25% Tween-20

- 1 x TBST:

  5 x TBST: deionized H_2O (1:4)

- 3% (w/v) bovine serum albumin (BSA) in TBST
- 1% (w/v) BSA in TBST

b) Procedure

Each PVDF membrane containing proteins was placed into a clean black blotting box and incubated with 10 mL of 3% BSA-TBST on a shaker at 18-22°C for 1-2 hours. Primary antibodies were diluted 1:1000 in 3% BSA (to equal a 10 mL total volume) and the solution was incubated with the membrane for 60 min on a shaker at 18-22°C. Membranes were subsequently washed four times with 1 x TBST (10 mL/wash) and incubated for an additional 60 min on a shaker at 18-22°C in 1% BSA-TBST with diluted HRP-coupled secondary antibody (1:10 000). After the membranes had been washed four times with 1 x TBST (10 mL/wash), HRP was detected using the ECL chemiluminescent system.

Detection of HRP in PVDF membranes

Horseradish peroxidase (HRP)-conjugated antibodies bound to immobilized antigens (i.e. proteins) were detected using ECL and ECL Plus Western Blotting Detection Reagents (Amersham Biosciences). The ECL Detection systems are based on the principle of chemiluminescence where oxidation reactions in the presence of HRP result in the emission of light that can be detected by exposure to film. The ECL Western Blotting Detection Reagents are based on the oxidation of the cyclic diacylhydrazide, luminol, whereas the ECL Plus detection system is based on the enzymatic generation of an acridinium ester, which produces a more intense light emission of longer duration.

a) Reagents

ECL Western Blotting Detection Reagents (RPN2109):

Detection reagent 1

Detection reagent 2

ECL Plus Western Blotting Detection Reagents (RPN2132):

Detection reagent A

Detection reagent B

b) Procedure

The luminescent reagent was prepared from the detection reagents in a clean blotting box. The PVDF membrane was removed from the last wash, placed in the reagent and agitated for approximately 30 – 60 seconds. The inside of two acetate sheets were cleaned with deionized water and dried. The membrane was removed from the luminescent reagent; shaken to remove excess liquid and

placed between the acetate sheets. Excess liquid and air trapped between sheets was removed and the acetate was exposed to audioradiographic film. Quantification of data obtained on film (Kodak X-omat) was accomplished with a GS800 Imaging Densitometer (Bio-Rad Laboratories, Missisauga, ON) under nonsaturating conditions with local background subtraction. Although multiple exposures were acquired to ensure the absence of film saturation, the experimental figures typically show longer exposures selected specifically for visual presentation and not necessarily used for data analysis.

Appendix B: Supplementary tables for manuscripts

Chapter 5:

Table B-1. Urinary excretion of D-*chiro*-inositol, *myo*-inositol, glucose and glucuronic acid in individuals with and without diabetes. ***(Figure 5-1)***

Chapter 6:

Table B-2. Effect of an acute dose of a buckwheat concentrate on serum glucose concentrations during an intraperitoneal glucose tolerance test of Sprague-Dawley rats. ***(Figure 6-2)***

Table B-3. Effect of an acute dose of a buckwheat concentrate on serum insulin concentrations during an intraperitoneal glucose tolerance test of Sprague-Dawley rats.

Table B-4. Effect of an acute dose of a buckwheat concentrate on serum glucose concentrations during an intraperitoneal glucose tolerance test of fed STZ rats. ***(Figures 6-3 and 6-4)***

Table B-5. Effect of a buckwheat concentrate or placebo on serum glucose concentrations during an oral glucose tolerance test in fasted STZ rats. ***(Figures 6-5 and 6-6)***

Chapter 7:

Table B-6. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of select signal transduction proteins in H4IIE cells. ***(Figures 7-2, 7-6, 7-7)***

Table B-7. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on stimulation of mitogen-activated protein kinase (MAPK) in H4IIE cells. ***(Figure 7-3)***

Table B-8. The concentration-dependent effects of the buckwheat concentrate (BWC) on mitogen-activated protein kinase (MAPK) phosphorylation. ***(Figure 7-4)***

Table B-9. The concentration-dependent effects of insulin (INS) on mitogen-activated protein kinase (MAPK) phosphorylation.

Table B-10. The effects of insulin (INS), the buckwheat concentrate (BWC), and their additive effects on phosphorylation of mitogen-activated protein kinase (MAPK) in H4IIE cells. ***(Figure 7-5)***

Table B-11. The effects of inhibitors of phopholipase D (butanol), Src kinase (PP1), and Arf3 (brefeldin) on insulin stimulated phosphorylation of select signal transduction proteins. ***(Figure 7-8)***

Table B-12. The effects of inhibitors of phopholipase D (butanol), Src kinase (PP1), and Arf3 (brefeldin) on the buckwheat concentrate (BWC) stimulated phosphorylation of select signal transduction proteins. ***(Figure 7-8)***

Chapter 8:

Table B-13. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on uptake of ^{3}H-deoxy-glucose in H4IIE cells. ***(Figure 8-1)***

Table B-14. The dose-dependent effects of the buckwheat concentrate on uptake of ^{3}H-deoxy-glucose in H4IIE cells. ***(Figure 8-2)***

Table B-15. The effects of the buckwheat concentrate (BWC) on basal and insulin-stimulated uptake of ^{3}H-deoxy-glucose in H4IIE cells. ***(Figure 8-3)***

Table B-16. The effects of inhibiting the phosphorylation of MAPK by PD98059, insulin receptor tyrosine kinase by AG1024 and phospholipase D by 1-butanol on insulin (INS)-stimulated uptake of ^{3}H-deoxy-glucose in H4IIE cells. ***(Figure 8-4)***

Chapter 7 and 8:

Table B-17. Soluble carbohydrates present in the buckwheat concentrate (BWC) used for cell culture experiments.

Table B-1. Urinary excretion of D-*chiro*-inositol, *myo*-inositol, glucose and glucuronic acid in individuals with and without diabetes.

	Myo-inositol (μM/24 hrs)	D-*chiro*-inositol (μM/24 hrs)	Glucose (mM/24 hrs)	Glucuronic acid (μM/24 hrs)
DM-1	757 ± 132^{B}	308 ± 133^{A}	53.4 ± 16.0^{B}	2050 ± 552^{B}
DM-1 Control	102 ± 20^{B}	19.1 ± 7.9^{A}	0.11 ± 0.04^{B}	148 ± 41^{B}
DM-2	227 ± 96^{B}	31.0 ± 15.1^{A}	0.10 ± 0.05^{B}	249 ± 76^{B}
DM-2 Control	157 ± 29^{B}	32.8 ± 10.8^{A}	0.01 ± 0.00^{B}	198 ± 57^{B}

Means with different superscript letters are significantly different as determined by Duncan's multiple range test. Data provided in Table B-1 correspond to Figure 5-1.

Table B-2. Effect of an acute dose of a buckwheat concentrate on serum glucose concentrations during an intraperitoneal glucose tolerance test of Sprague-Dawley rats.[1]

Time (minutes)	Treatment Groups [2]	
	SDC	SDBW
0	7.36 ± 0.43	6.88 ± 0.31
30	38.55 ± 0.94^{A}	31.91 ± 2.38^{B}
60	26.88 ± 4.36	22.09 ± 5.33
90	11.06 ± 1.47	10.39 ± 0.90
120	11.13 ± 0.88	9.91 ± 0.51

[1] Serum glucose values are expressed as mmol/L. Values are means ± SE. Superscript letters indicate significant differences between means as determined by Duncan's multiple range test. Data provided in Table B-2 correspond to Figure 6-2.

[2] SDC=Sprague-Dawley control, SDBW=Sprague-Dawley acute dose buckwheat concentrate. Treatment group details are provided in Chapter 6.

Table B-3. Effect of an acute dose of a buckwheat concentrate on serum insulin concentrations during an intraperitoneal glucose tolerance test of Sprague-Dawley rats.[1]

Time (minutes)	Treatment Groups [2]	
	SDC	SDBW
0	1.47 ± 0.10	1.75 ± 0.32
30	6.63 ± 0.69 [3]	6.28 ± 0.90 [3]
60	5.58 ± 0.53 [6]	4.08 ± 1.65 [5]
90	2.27 ± 0.24 [4]	1.92 ± 0.08 [5]
120	2.06 ± 0.18 [3]	1.44 ± 0.23 [3]

[1]Serum insulin values are expressed in ng/mL. Values are means ± SE. There were no significant differences between means determined by Duncan's multiple range test.
[2] SDC=Sprague-Dawley control, SDBW=Sprague-Dawley acute dose buckwheat concentrate. n=6 per group unless otherwise indicated. Treatment group details are provided in Chapter 6.
[3] n=5 due to insufficient amounts of serum collected.
[4] n=4 due to insufficient amounts of serum collected.
[5] n=3 due to insufficient amounts of serum collected.
[6] n=2 due to insufficient amounts of serum collected.

Table B-4. Effect of an acute dose of a buckwheat concentrate on serum glucose concentrations during an intraperitoneal glucose tolerance test of fed STZ rats.[1]

Time (minutes)	Treatment			
	BW LOW	PL LOW	BW HIGH	PL HIGH
0	30.2 ± 1	26.7 ± 1.3	29.6 ± 1.4	29.6 ± 0.7
30	29.7 ± 1.5	28.3 ± 0.9	31.4 ±1	32.6 ± 0.8
60	25.82 ± 1.3	28.9 ± 1.8	29.2 ± 0.7	30.2 ± 0.8
90	26.0 ± 1.2	28.8 ± 1.7	25.6 ± 0.9*	29.8 ± 0.6
120	25.4 ± 1.4	27.6 ± 1.3	23.8 ± 0.5**	28.9 ± 0.6

[1]Serum glucose is expressed as mmol/L. Values expressed are means ± SE. BW LOW = received a low dose, 10 mg free D-CI/kg body weight, of the buckwheat concentrate (n=8); PL LOW = low dose placebo (n=9); BW HIGH = received a high dose, 20 mg free D-CI/kg body weight, of the buckwheat concentrate (n=9); PL HIGH = high dose placebo (n=10). Treatment group details are provided in Chapter 6. Data provided in Table B-4 correspond to Figures 6-3 and 6-4.

*Results from the t-test determined the value was significantly lower than the PL HIGH group at $p<0.002$.

**Results from the t-test determined the value was significantly lower than the PL HIGH group at $p<0.0001$.

Table B-5. Effect of a buckwheat concentrate or placebo on serum glucose concentrations during an oral glucose tolerance test in fasted STZ rats.[1]

Time (minutes)	Treatment			
	BW LOW	PL LOW	BW HIGH	PL HIGH
0	24.7 ± 1.4	24.4 ± 1.4	25.8 ± 1.1	27.4 ± 1.4
30	28.7 ± 1.5	30 .9 ± 2.3	28.3 ± 1.2	29.9 ± 1.6
60	26.5 ± 1.3	27.9 ± 1.9	27.1 ± 0.9	29.5 ± 1.4
90	24.7 ± 1.1	26.1 ± 1.5	25.3 ± 0.7	27.5 ± 1.7
120	23.7 ± 1.5	25.7 ± 1.6	24.5 ± 0.9	26.7 ± 1.3

[1]Serum glucose is expressed as mmol/L. Values expressed are means ± SE. BW LOW = received a low dose, 10 mg free D-CI/kg body weight, of the buckwheat concentrate (n=8); PL LOW = low dose placebo (n=9); BW HIGH = received a high dose, 20 mg free D-CI/kg body weight, of the buckwheat concentrate (n=9); PL HIGH = high dose placebo (n=10). Treatment group details are provided in Chapter 6. No significant differences were determined by the t-test. Data provided in Table B-5 correspond to Figures 6-5 and 6-6.

Table B-6. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of select signal transduction proteins in H4IIE cells.

Antibody	Ctrl	Insulin	BWC	D-CI	MI
InsR (Tyr^{1146})	0.47 ± 1.47	10.1 ± 1.47*	0.43 ± 1.47	0.71 ± 1.47	0.96 ± 1.47
GSK3 ($Ser^{21/9}$)	0.52 ± 0.17	2.42 ± 0.17*	0.21 ± 0.17	0.24 ± 0.17	0.02 ± 0.17
Src (Tyr^{527})	0.05 ± 0.64	5.29 ± 0.64*	0.05 ± 0.64	0.13 ± 0.64	0.03 ± 0.64
Stat3 (Tyr^{705})	0.19 ± 0.69	2.74 ± 0.69	0.04 ± 0.69	0.11 ± 0.69	0.15 ± 0.69
Akt (Ser^{473})	0.89 ± 0.48	28.4 ± 0.48*	1.17 ± 0.48	1.12 ± 0.48	1.10 ± 0.48
PDK-1 (Ser^{241})	2.22 ± 1.35	2.63 ± 1.35	2.09 ± 1.35	2.00 ± 1.35	2.40 ± 1.35
ELK-1 (Ser^{383})	4.99 ± 3.74	5.40 ± 3.74	6.35 ± 3.74	5.05 ± 3.74	6.47 ± 3.74
Raf (Ser^{259})	10.2 ± 4.2	10.9 ± 4.2	9.71 ± 4.24	10.8 ± 4.2	10.6 ± 4.2
PRK1 (Thr^{778})	1.97 ± 0.86	1.48 ± 0.86	1.14 ± 0.86	1.57 ± 0.86	1.85 ± 0.86
AMPK (Ser^{108})	2.29 ± 0.52	1.57 ± 0.52	1.76 ± 0.52	0.84 ± 0.52	0.92 ± 0.52
IRS-1 (Tyr)	0.46 ± 0.32	1.68 ± 0.32	0.72 ± 0.32	0.72 ± 0.32	0.42 ± 0.32

H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (Ctrl). Each experiment was replicated three times and protein phosphorylation in cellular extracts was determined by Western blotting. Treatment and antibody specifications are provided in Chapter 7. Band intensities on each blot were quantified by scanning densitometry and are presented as means ± SE (n=3). Statistical significance ($p<0.05$) was determined by a mixed model analysis with a random intercept to account for differences between repetitions. Estimate statements were used to determine differences between treatments versus the control and are indicated (*). Data provided in Table B-6 correspond to Figures 7-2, 7-6 and 7-7.

Table B-7. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on stimulation of mitogen-activated protein kinase (MAPK) in H4IIE cells.

Antibody	Ctrl	Insulin	BWC	D-CI	MI
MAPK	12.8 ± 7.0	13.8 ± 7.0	11.7 ± 7.0	12.1 ± 7.0	13.1 ± 7.0
P-MAPK	0.05 ± 1.00	10.0 ± 1.0*	8.09 ± 1.00*	0.04 ± 1.00	0.04 ± 1.00

H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control. Each experiment was replicated three times. Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) were determined by Western blotting. Treatment and antibody specifications are provided in Chapter 7. Band intensities on each blot were quantified by scanning densitometry and are presented as means ± SE (n=3). Statistical significance ($p<0.05$) was determined by a mixed model analysis with a random intercept to account for differences between repetitions. Estimate statements were used to determine differences between treatments versus the control are indicated (*). Data provided in Table B-7 correspond to Figure 7-3.

Table B-8. The concentration-dependent effects of the buckwheat concentrate (BWC) on mitogen activated protein kinase (MAPK) phosphorylation.

Antibody	BWC (μL)						
	0	0.5	1	2	5	10	20
MAPK	14.2 ± 4.4	12.9 ± 4.4	12.6 ± 4.4	10.8 ± 4.4	10.4 ± 4.4	9.8 ± 4.4	10.4 ± 4.4
P-MAPK	1.94 ± 1.40	2.01 ± 1.40	2.64 ± 1.40	3.09 ± 1.40	9.44 ± 1.40*	8.44 ± 1.40*	12.0 ± 1.40*

H4IIE cells were treated with each concentration individually for 6 minutes, with untreated cells serving as the control (0 μL BWC). Each experiment was replicated three times. Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) were determined by Western blotting. Treatment and antibody specifications are provided in Chapter 7. Band intensities on each blot were quantified by scanning densitometry and are presented as means ± SE (n=3). Statistical significance ($p<0.05$) was determined by a mixed model analysis with a random intercept to account for differences between repetitions. Estimate statements were used to determine differences between treatments versus the control and are indicated (*). Data provided in Table B-8 correspond to Figure 7-4.

Table B-9. The concentration-dependent effects of insulin on mitogen-activated protein kinase (MAPK) phosphorylation.

Antibody	Insulin (nM)						
	0	2.5	5	10	25	50	100
MAPK	14.7 ± 6.2	14.5 ± 6.2	14.9 ± 6.2	15.2 ± 6.2	13.8 ± 6.2	14.6 ± 6.2.	15.6 ± 6.2
P-MAPK	0.29 ± 0.17	0.35 ± 0.17	0.66 ± 0.17	0.70 ± 0.17	0.79 ± 0.17*	0.85 ± 0.17*	0.53 ± 0.17

H4IIE cells were treated with each concentration individually for 6 minutes, with untreated cells serving as the control (0 nM insulin). Each experiment was replicated three times. Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) were determined by Western blotting. Treatment and antibody specifications are provided in Chapter 7. Band intensities on each blot were quantified by scanning densitometry and are presented as means ± SE (n=3). Statistical significance ($p<0.05$) was determined by a mixed model analysis with a random intercept to account for differences between repetitions. Estimate statements were used to determine differences between treatments versus the control and are indicated (*).

Table B-10. The effects of insulin (INS), the buckwheat concentrate (BWC), and their additive effects on phosphorylation of mitogen-activated protein kinase (MAPK) in H4IIE cells.

Antibody	Insulin	BWC	BW + INS	INS + BW
MAPK	1.49	1.47	0.99	0.80
P-MAPK	123	119	215	228
RATIO	82.8	81.4	217	286

H4IIE cells were treated for 6 minutes with INS, BWC or a combination of both. Either INS (I+BW) or BWC (BW+I) was added first. Untreated cells served as the control. Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) were determined by Western blotting. The ratio of P-MAPK/MAPK is also presented (RATIO). Treatment and antibody specifications are provided in Chapter 7. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00. Data presented is from one experiment. Data provided in Table B-10 correspond to Figure 7-5.

Table B-11. The effects of inhibitors of phospholipase D (butanol), Src kinase (PP1), and Arf3 (brefeldin) on insulin-stimulated phosphorylation of select signal transduction proteins.

Antibody	Inhibitors				
	Ctrl	None	Brefeldin	1-butanol	PP1
MAPK	12.8 ± 4.9	13.5 ± 4.9	12.8 ± 4.9	12.3 ± 4.9	14.0 ± 4.9
P-MAPK	0.02 ± 0.80	2.39 ± 0.80*	2.04 ± 0.80	0.70 ± 0.80	2.56 ± 0.80*
InsR (Tyr^{1146})	1.34 ± 1.22	10.7 ± 1.2*	12.2 ± 1.2*	12.0 ± 1.2*	9.46 ± 1.22*
GSK3 ($Ser^{21/9}$)	0.57 ± 0.92	2.28 ± 0.92	2.04 ± 0.92	2.05 ± 0.92	2.43 ± 0.92

Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with insulin for 6 minutes with untreated cells serving as the control (Ctrl). Extracts were subsequently prepared for Western blot analysis. Treatment and antibody specifications are provided in Chapter 7. Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and are presented as means ± SE (n=3). Statistical significance ($p<0.05$) was determined by a mixed model analysis with a random intercept to account for differences between repetitions. Estimate statements were used to determine differences between treatments versus the control and are indicated (*). Data provided in Table B-11 correspond to Figure 7-8.

Table B-12. The effects of inhibitors of phospholipase D (butanol), Src kinase (PP1), and Arf3 (brefeldin) on the buckwheat concentrate (BWC)-stimulated phosphorylation of selected signal transduction proteins.

Antibody	Inhibitors				
	Ctrl	None	Brefeldin	1-butanol	PP1
MAPK	12.8 ± 5.3	14.5 ± 5.3	13.7 ± 5.3	13.1 ± 5.3	14.0 ± 5.3
P-MAPK	0.02 ± 0.88	3.34 ± 0.88*	2.88 ± 0.88*	0.49 ± 0.88	0.73 ± 0.88
InsR (Tyr^{1146})	1.34 ± 0.67	1.66 ± 0.67	1.36 ± 0.67	1.33 ± 0.67	1.04 ± 0.67
GSK3 ($Ser^{21/9}$)	0.57 ± 0.23	0.63 ± 0.23	0.87 ± 0.23	0.30 ± 0.23	0.28 ± 0.23

Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with the BWC for 6 minutes, with untreated cells serving as the control (Ctrl). Extracts were subsequently prepared for Western blot analysis. Treatment and antibody specifications are provided in Chapter 7. Each experiment was replicated three times. Band intensities on each blot were quantified by scanning densitometry and are presented as means ± SE (n=3). Statistical significance ($p<0.05$) was determined by a mixed model analysis with a random intercept to account for differences between repetitions. Estimate statements were used to determine differences between treatments versus the control and are indicated (*). Data provided in Table B-12 correspond to Figure 7-8.

Table B-13. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on uptake of ^{3}H-deoxy-glucose in H4IIE cells.

Treatment	^{3}H-deoxyglucose uptake (CPM)
Control	306 ± 53^{B}
Insulin	570 ± 117^{A}
BWC	76.5 ± 11.5^{C}
D-CI	585 ± 97^{A}
MI	420 ± 57^{BC}

Treatments were either INS (250 nM), BWC, D-CI, or MI (2 μL or a final concentration of 0.4% (v/v)). Experimental details are provided in Chapter 8. The data are presented as means ± SE for three independent experiments. Statistical significance ($p<0.05$) was determined by one-way ANOVA. Means with different superscipt letters are significantly different as determined by Duncan's mutiple range test ($p<0.05$). Data provided in Table B-13 correspond to Figure 8-1.

Table B-14. The dose-dependent effects of the buckwheat concentrate (BWC) on uptake of ^{3}H-deoxy-glucose in H4IIE cells.

Treatment	^{3}H-deoxyglucose uptake (CPM)
Control	196 ± 32
BWC 0.5 μL	103 ± 8
BWC 2 μL	97.3 ± 23.6
BWC 10 μL	196 ± 87

Treatments were either 0.5 μL, 2 μL or 10 μL of the BWC (final concentrations of 0.1%, 0.4%, or 2% (v/v), respectively). Experimental details are provided in Chapter 8. The data are presented as means ± SE for three independent experiments. There were no statistically significant differences between groups as determined by ANOVA. Data provided in Table B-14 correspond to Figure 8-1.

Table B-15. The effects of the buckwheat concentrate (BWC) on basal and insulin-stimulated uptake of ^{3}H-deoxy-glucose in H4IIE cells.

Treatment	^{3}H-deoxyglucose uptake (CPM)
Control	196 ± 32^{A}
Insulin	594 ± 135^{B}
BWC 2 μL	97.3 ± 23.6^{A}
INS + BWC	78.3 ± 11.5^{A}

Treatments were either INS (250 nM), BWC (2 μL or a final concentration of 0.4% (v/v)), or insulin plus buckwheat added together (INS + BWC). Experimental details are provided in Chapter 8. The data are presented as means ± SE for three independent experiments. Statistical significance ($p<0.05$) was determined by one-way ANOVA. Means with different superscipt letters are significantly different as determined by Duncan's mutiple range test ($p<0.05$). Data provided in Table B-15 correspond to Figure 8-3.

Table B-16. The effects of inhibiting the phosphorylation of MAPK by PD98059, insulin receptor tyrosine kinase by AG1024 and phospholipase D by 1-butanol on insulin (INS)-stimulated uptake of ^{3}H-deoxy-glucose in H4IIE cells.

Treatment	^{3}H-deoxyglucose uptake (CPM)
Control	453 ± 36^{AB}
Insulin	727 ± 332^{A}
INS + PD98059	105 ± 26^{B}
INS + AG1024	55.8 ± 23.0^{B}
INS + 1-butanol	359 ± 80^{AB}

Treatments included insulin (INS) (250 nM) ± inhibitors as described in Chapter 8. The data are presented as means ± SE for three independent experiments. Statistical significance ($p<0.05$) was determined by one-way ANOVA. Means with different superscipt letters are significantly different as determined by Duncan's mutiple range test ($p<0.05$). Data provided in Table B-16 correspond to Figure 8-4.

Table B-17. Soluble carbohydrates present in the buckwheat concentrate used for cell culture experiments.[1]

Component	Concentration (mg/mL)
fructose	122
glucose	107
D-*chiro*-inositol	10.3
myo-inositol	10
sucrose	158
raffinose	2
stachyose	2

[1] Composition of the buckwheat concentrate used for cell culture experiments in Chapters 7 and 8. Soluble carbohydrates were analyzed by gas chromatography as described in Chapter 6 (Section 6.3).

Appendix C: Additional experiments evaluating the effects of buckwheat supplementation in animal models

Introduction

In addition to the experiments involving acute administration of a buckwheat concentrate (Chapter 6), the efficacy of chronic administration of a buckwheat concentrate was evaluated in animal models of DM-2. The effects of dietary supplementation with buckwheat concentrates on serum glucose concentrations were determined in diabetic db/db mice (a DM-2 model) and fa/fa Zucker rats (a model of IGT).

C57BL/6J and db/db mice

Female weanling C57BL/6J mice and C57BL/6J homozygous diabetic (db/db) mice were purchased from the Jackson Laboratory (Bar Harbour, Maine). Prior to initiating a 6 week buckwheat supplementation period in db/db mice, a pilot study was performed using C57BL/6J mice, the background strain of db/db mice, to ensure mice would consume buckwheat supplemented diets. Upon arrival, 18 C57BL/6J mice were randomly assigned to one of three treatment groups (described below). Each treatment group was initially fed the control diet for a 5-day acclimatization period. Mice were then fed ad libitum dietary treatments for a period of 2 weeks. Weekly body weight and daily feed intake was recorded. Upon arrival, 24 C57BL/6J homozygous diabetic (db/db) mice were randomly assigned to one of three treatment groups. Each treatment group was initially fed the control diet for a 7 to 9 day acclimatization period. Mice were then fed *ad libitum* dietary treatments for a period of 6 weeks. Weekly body weight and daily feed intake was recorded.

Dietary treatments based on the AIN-93G formulation (Reeves, 1993) included a control diet and diets supplemented with either a low dose (0.05 mg/g diet) or high dose (0.25 mg/g diet) of D-*chiro*-inositol in the form of a concentrate produced from buckwheat. Diet formulation and buckwheat supplementation details are provided in Table C-1. The powder mix of dry ingredients was used to make a paste diet as described in Table C-1. Mice were maintained in a controlled environment of 21-23°C, 55% humidity, and a 14 hour light, 10 hour dark cycle. During the feeding trials, the mice were housed separately in stainless steel hanging cages and fresh water was available in polypropylene bottles with stainless steel sipper tubes. At weeks 0, 3, and 5.5 of trial, db/db mice were housed overnight (12 hours) in polycarbonate metabolic cages (Nalgene, Fisher Scientific) for the purpose of urine collection. Urine was also collected from C57BL/6J mice at week 2. Animals were provided water ad libitum (but not feed) for the 12 hours to ensure urine samples were not contaminated by the feed. After an overnight fast at week 2 for C57BL/6J mice and weeks 0, 3, and 5.5 for db/db mice, urine samples were collected in 1.5 mL microcentrifuge tubes from the metabolic cages and weighed to determine volume. All urine samples were stored at -20° C until assayed for glucose, creatinine and inositols. Methods for analysis are described in Chapter 4 and Appendix A.

C57BL/6J mice were terminated at week 2 following urine collection. The db/db mice were terminated at week 6 following a 6 hour fast. Animals were terminated by CO_2 asphyxiation and cervical dislocation following Canadian

Council on Animal Care Guidelines. Trunk blood was collected into plastic tubes after decapitation and samples were stored on ice until centrifuged. The liver and kidneys were excised from C57BL/6J mice and immediately frozen in liquid nitrogen. The liver, pancreas, kidneys, heart, small intestine, parametrical fat pads and peri-renal fat pads were excised from db/db mice and immediately frozen in liquid nitrogen. All tissue samples and serum were stored at -80° C. Serum concentrations of glucose and insulin were determined as described in Appendix A.

Zucker rats

Male weanling fa/fa and lean Zucker rats were purchased from Charles River. Upon arrival, 12 fa/fa and 15 lean rats were randomly assigned to one of three treatment groups. Each treatment group was initially fed the control diet for a 4-6 day acclimatization period. Rats were then fed ad libitum dietary treatments for a period of 3 weeks. Weekly body weight and daily feed intake was recorded.

Dietary treatments were based on the AIN-93G formulation (Reeves, 1993). Treatments included a control diet and diets supplemented with D-*chiro*-inositol (0.12 mg/g diet) in the form of either a concentrated powder produced from dehulled whole buckwheat or a concentrated powder produced from milled buckwheat. Buckwheat supplementation details are provided in Table C-2. Soluble carbohydrates were extracted from either dehulled whole buckwheat or a milled fraction of buckwheat and analyzed as described in Chapter 6. Extracts were then freeze-dried under a vacuum and added to diet formulations as

described in Table C-2. Rats were maintained in a controlled environment of 21-23°C, 55% humidity, and a 14 hour light, 10 hour dark cycle. During the feeding trials, the rats were housed separately in stainless steel hanging cages and fresh water was available in polypropylene bottles with stainless steel sipper tubes.

Oral glucose tolerance testing was done at week 2.5. Training was conducted 3-5 days prior to testing to familiarize animals with the procedure. For the oral glucose tolerance test, blood was collected for the 0 minute time point from the saphanous vein and immediately following, a 70% glucose solution (7g glucose/10 mL deionized water) was administered orally with a syringe (1 g glucose/kg body weight). Blood was collected at 15, 30, 60 and 90 minutes following administration of the glucose solution. Blood was held on ice until centrifuged to obtain serum.

At weeks 0 and 3, rats were housed overnight (12 hours) in polycarbonate metabolic cages (Nalgene, Fisher Scientific) for the purpose of urine collection. Animals were provided water ad libitum (but not feed) to ensure urine samples were not contaminated by the feed. Urine samples were collected into scintillation vials from the metabolic cages and weighed to determine volume. All urine samples were stored at -20°C until assayed for glucose, creatinine and inositols as described in Chapter 4 and Appendix A. At week 3, fasting blood samples were obtained from the saphenous vein of fa/fa rats following urine collection and prior to termination. All animals were terminated by CO_2 asphyxiation and cervical dislocation following Canadian Council on Animal Care Guidelines. Trunk blood was collected into plastic tubes after decapitation and

samples were stored on ice until centrifuged to obtain serum. The liver, pancreas, kidneys, heart, aorta, small intestine, epididymal fat pads, peri-renal fat pads, subcutaneous fat, and gastrocnemius muscle were excised and immediately frozen in liquid nitrogen. All tissue samples and serum were stored at -80°C until required for analysis. Serum samples were analyzed for concentrations of glucose and insulin as described in Appendix A.

Summary of results

In the present research, we evaluated the effects of chronic supplementation with a buckwheat concentrate on glycemic control in the diabetic db/db mouse and fa/fa Zucker rat. The main findings of these studies were that 1) dietary supplementation with two doses of a buckwheat concentrate (approximately 15 mg D-CI/kg body weight/day or 75 mg D-CI/kg body weight/day) for 6 weeks in the db/db mouse had no effect on fasting serum glucose or insulin concentrations, 2) dietary supplementation with a buckwheat concentrate produced either from whole buckwheat or milled buckwheat (approximately 10-15 mg D-CI/kg body weight/day) for 3 weeks in fa/fa Zucker rats had no effect on fasting serum glucose or on oral glucose tolerance, 3) the buckwheat supplemented diets were tolerated and nontoxic in both animal models and 4) D-*chiro*-inositol (D-CI) from buckwheat was absorbed and excreted as indicated by the elevated urinary D-CI excretion observed in buckwheat supplemented-fa/fa and lean Zucker rats compared to the control animals, whereas *myo*-inositol excretion was not elevated in buckwheat-supplemented rats.

Table C-1. Diet formulations used for buckwheat supplementation studies in C57BL/6J and db/db mice.[1]

Ingredient	Amount (g/kg powder diet)
Dextrose (cerelose)[2]	594.6
Egg white	212.5
Fiber (cellulose)	50
Mineral mix (zinc-free) (AIN-93)	35
Potassium PO_4	5.4
Vitamin mix (AIN-93)	10
Choline bitartrate	2.5
Biotin mix[3]	10
Zinc premix[4]	10
Soybean oil[5]	70

[1]Powder mix of dry ingredients was used to make a paste diet (200 mL water or a buckwheat extract was added to 1 kg dry diet to yield 1200 g paste diet). For the low-dose buckwheat diet (0.05 mg D-*chiro*-inositol/g diet), the buckwheat extract contained 60 mg of D-*chiro*-inositol/200 mL. For the high-dose buckwheat diet (0.5 mg/g diet), the buckwheat extract contained 600 mg D-*chiro*-inositol/200 mL. All diet ingredients were purchased from Harlan Teklad (Madison, WI) unless otherwise indicated.
[2]Dextrose was purchased from Moonshiners (Winnipeg, MB).
[3]Biotin mix (200 mg/kg mix)
[4] Zinc premix (5.81 g Zn carbonate/1000 g dextrose)
[5]Soybean oil was purchased from Vita Health (Winnipeg, MB).

Table C-2. Diet formulations used for buckwheat supplementation studies in fa/fa and lean Zucker rats.[1]

Ingredient	Amount (g/kg diet)
Cornstarch	347.6
Maltodextrin	132
Sucrose	100
Egg white	212.5
Fiber (cellulose)	50
Mineral mix (zinc-free)	35
Potassium phosphate	5.4
Vitamin mix	10
Choline	2.5
Biotin mix (200 mg/kg mix)[2]	10
Zinc premix[3]	10
Tert-butylhydroquinone	0.014
Soybean Oil[4]	85

[1]Buckwheat extracts produced from whole buckwheat (BWCN) and milled buckwheat (BWML) were freeze dried and the resulting powder was added to the diets to provide 0.12 mg D-*chiro*-inositol per gram of diet. The total amount of powder added the diet was 16 g/kg diet and the composition of the powder was 6.8 – 7.5 mg D-*chiro*-inositol/g powder for BWCN and BWML. All diet ingredients were purchased from Harlan Teklad (Madison, WI) unless otherwise indicated.
[2]Biotin mix (200 mg/kg mix in cornstarch)
[3]Zinc premix (5.775 g Zn carbonate + 994.2 g cornstarch)
[4]Soybean oil was purchased from Vita Health (Winnipeg, MB)

Results for C57BL/6J mice

Table C-3. Effect of dietary supplementation with a buckwheat concentrate for 2 weeks on body weight, feed intake, and serum glucose of C57BL/6J mice.[1]

	Treatment Groups[2]		
	CC	CBWL	CBWH
Final body weight[3] (g)	20.5 ±0.41	21.4 ±0.61	20.8 ±0.33
Total feed intake[4] (g)	60.2 ±0.96	63.3 ±1.90	62.0 ±0.88
Liver weight (g)	0.81 ±0.02	0.79 ±0.02	0.76 ±0.03
Serum glucose (mmol/L)	7.55 ±0.61	7.81 ±0.83	8.98 ±0.65

[1] Values are means (n=6) ± SE. There were no significant differences as determined by an ANOVA.
[2] CC = C57BL/6J control, CBWL = C57BL/6J low dose buckwheat concentrate supplement, CBWH = C57BL/6J high dose buckwheat concentrate supplement.
[3] Body weight at week 2.
[4] Total feed intake for 2 weeks.

Results for db/db mice

Table C-4. Effect of dietary supplementation with a buckwheat concentrate for 6 weeks on body weight and feed intake of db/db mice.[1]

	Treatment Groups [2]		
	dbC	dbBWL	dbBWH
Body weight week 0	25.2 ±1.28	19.7 ±0.83	21.9 ±0.77
Body weight week1	29.2 ±1.13	24.8 ±0.83	25.6 ±0.93
Body weight week 2	27.9 ±1.22	27.7 ±0.82	27.9 ±0.92
Body weight week 3	29.6 ±1.11	29.1 ±0.74	29.4 ±0.74
Body weight week 4	30.1 ±1.11	29.3 ±0.70	30.0 ±0.83
Body weight week 5	30.6 ±1.06	29.2 ±1.01	30.7 ±0.98
Body weight week 6	30.4 ±1.02	27.6 ±1.07	29.7 ±1.16
Total feed intake[3]	316.2 ±15.8	344.3 ±13.5	325.9 ±24.7

[1] Values provided are in grams and are means ± SE. n=8 except n=7 for dbBWL. There were no significant differences as determined by an ANOVA.
[2] dbC = db/db control, dbBWL = db/db low dose buckwheat concentrate supplement, dbBWH = high dose buckwheat concentrate supplement.
[3] Total feed intake for 6 weeks.

Table C-5. Effect of dietary supplementation with a buckwheat concentrate for 6 weeks on fasting body weight and tissue weights of db/db mice.[1]

	Treatment Groups [2]		
	dbC	dbBWL	dbBWH
Fasting body weight (g)	29.3 ±1.05	26.7 ±1.09	28.8 ±1.17
Pancreas weight (g)	0.26 ±0.02	0.25 ±0.02	0.29 ±0.01
Liver weight (g)	1.59 ±0.09	1.50 ±003	1.53 ±006
Parametrical fat pad weight (g)	1.59 ±0.07	1.30 ±0.06	1.52 ±0.3
Kidneys weight (g)	0.41 ±0.02	0.42 ±0.02	0.44 ±0.02
Peri-renal fat pad weight (g)	0.92 ±0.07	0.90 ±0.06	0.97 ±0.06
Heart weight (g)	0.14 ±0.01	0.13 ±0.01	0.12 ±0.00
Small intestine weight (g)	1.25 ±0.04	1.24 ±0.08	1.31 ±0.08
Small intestine length (cm)	35.1 ±0.7	33.1 ±1.9	33.8 ±1.5

[1] Values are means ± SE. n=8 except n=7 for dbBWL. There were no significant differences between means as determined by an ANOVA.
[2] dbC = db/db control, dbBWL = db/db low dose buckwheat concentrate supplement, dbBWH = high dose buckwheat concentrate supplement.

Table C-6. Effect of dietary supplementation with a buckwheat concentrate for 6 weeks on urine glucose of db/db mice.[1]

	Treatment Groups [2]		
	dbC	dbBWL	dbBWH
Week 0 (mg/12 hours)[3]	0.97 ±0.32	--	--
(mmol/L)[3]	0.48 ±0.07	--	--
Week 3 (mg/12 hours)[4]	23.2 ±6.36	34.0 ±9.89	40.8 ±13.8
(mmol/L)[4]	614.7 ±18.7	579.2 ±72.0	541.1 ±19.5
Week 5.5 (mg/12 hours)[5]	63.8 ±17.0	105.4 ±22.1	75.7 ±10.9
(mmol/L)[5]	625.6 ±36.3	753.1 ±63.4	666.2 ±49.5

[1] Values are means ± SE. n=8 for serum glucose and insulin, except n=7 for dbBWL. There were no significant differences as determined by an ANOVA.
[2] dbC = db/db control, dbBWL = db/db low dose buckwheat concentrate supplement, dbBWH = high dose buckwheat concentrate supplement.
[3] Due to the small amount of urine collected among individual treatment groups, 0 week urine glucose is the pooled mean (n=13) from all treatment groups.
[4] For 3 week urine glucose, n=7 for dbC and dbBWL and n=6 for dbBWH.
[5] For 5.5 week urine glucose, n=8 for dbC and dbBWH and n=6for dbBWL.

Table C-7. Effect of dietary supplementation with a buckwheat concentrate for 6 weeks on serum glucose and serum insulin of db/db mice.[1]

	Treatment Groups [2]		
	dbC	dbBWL	dbBWH
Serum glucose (mmol/L)	42.0 ±6.55	41.9 ±3.49	40.6 ±4.29
Serum insulin (ng/mL)	4.03 ±0.65	2.67 ±0.35	3.66 ±0.50

[1] Values are means ± SE. n=8 for serum glucose and insulin, except n=7 for dbBWL. There were no significant differences as determined by an ANOVA.
[2] dbC = db/db control, dbBWL = db/db low dose buckwheat concentrate supplement, dbBWH = high dose buckwheat concentrate supplement.

Table C-8. Correlations between serum insulin, feed intake, body weight, and urine glucose of db/db mice.[1]

	Treatment Groups[2]			
	Total	dbC	dbBWL	dbBWH
Serum insulin vs. Feed intake[3]	-0.676 (p=0.0004)	ns [5]	-0.852 (p=0.015)	-0.812 (p=0.015)
Serum insulin vs. Body weight[3]	ns	ns	0.817 (p=0.025)	0.628 (p=0.096)
Serum insulin vs. Urine glucose[4]	-0.550 (p=0.008)	ns	ns	-0.723 (p=0.043)
Feed intake vs. Urine glucose[4]	0.484 (p=0.022)	ns	ns	0.909 (p=0.002)

[1]Correlations are expressed as Pearson's correlation coefficients (*r*).
[2]Total=all treatment groups, dbC=db/db control, dbBWL=db/db low dose buckwheat concentrate supplement, dbBWH=db/db high dose buckwheat concentrate supplement.
[3]n=23 for total, n=7 for dbBWL, n=8 for dbBWH
[4]n=22 for total, n=8 for dbBWH
[5]ns=non-significant

Results for Zucker rats

Table C-9 . Effect of dietary supplementation with a buckwheat concentrate for 3 weeks on body weight and feed intake of fa/fa Zucker rats.[1]

	Treatment Groups [2]		
	faC	faBWCN	faBWML
Body weight week 0	207 ±4	206 ±6	204 ±4
Body weight week1	287 ±9	284 ±10	282 ±5
Body weight week 2	363 ±12	357 ±17	357 ±8
Body weight week 3	414 ±13	408 ±19	407 ±9
Total feed intake[3]	633 ±24	620 ±27	581 ±42

[1] Values provided are in grams and are means ± SE. n=4/treatment group. There were no significant differences between means as determined by an ANOVA.
[2] faC = fa/fa rats fed control diet, faBWCN = fa/fa rats fed whole buckwheat concentrate supplement, faBWML = fa/fa rats fed milled buckwheat concentrate supplement.
[3] Total feed intake for 3 weeks.

Table C-10. Effect of dietary supplementation with a buckwheat concentrate for 3 weeks on body weight and feed intake of lean Zucker rats.[1]

	Treatment Groups [2]		
	lnC	lnBWCN	lnBWML
Body weight week 0 (g)	132 ±7	135 ±9	126 ±2
Body weight week1 (g)	182 ±9	183 ±13	175 ±3
Body weight week 2 (g)	229 ±9	225 ±13	221 ±4
Body weight week 3 (g)	255 ±9	251 ±11	251 ±5
Total feed intake[3] (g)	375 ±15	373 ±14	368 ±9

[1] Values provided are in grams and are means ± SE. n=5/treatment group. There were no significant differences between means as determined by an ANOVA.
[2] lnC = lean rats fed control, lnBWCN = lean rats fed whole buckwheat concentrate supplement, lnBWML = lean rats fed milled buckwheat concentrate supplement.
[3] Total feed intake for 3 weeks.

Table C-11. Effect of dietary supplementation with a buckwheat concentrate for 3 weeks on fasting body weight and tissue weights of fa/fa Zucker rats.[1]

	Treatment Groups [2]		
	faC	faBWCN	faBWML
Fasting body weight	399 ±14	390 ±18	387 ±8
Pancreas	1.10 ±0.06	1.06 ±0.12	1.17 ±0.05
Liver	16.1 ±1.0	15.6 ±0.91	14.6 ±0.27
Gastrocnemius muscle	2.65 ±0.18	2.61 ±0.16	2.72 ±0.18
Peri-renal fat pad	11.5 ±0.3	12.2 ±0.4	11.1 ±0.5
Epididymal fat	10.9 ±0.4	10.2 ±0.5	11.6 ±0.3
Heart	1.12 ±0.07	1.13 ±0.04	1.08 ±0.03
Kidneys	2.70 ±0.08	2.99 ±0.27	2.54 ±0.09
Small intestine	6.47 ±0.26	6.98 ±0.16	6.67 ±0.25
Small intestine length (cm)	34.0 ±0.5	37.0 ±1.7	38.8 ±3.4

[1] Values provided are in grams except where indicated. Values are means ± SE. n=4/treatment group. There were no significant differences as determined by an ANOVA.

[2] faC = fa/fa rats fed control diet, faBWCN = fa/fa rats fed whole buckwheat concentrate supplement, faBWML = fa/fa rats fed milled buckwheat concentrate supplement.

Table C-12. Effect of dietary supplementation with a buckwheat concentrate for 3 weeks on fasting body weight and tissue weights of lean Zucker rats.[1]

	Treatment Groups [2]		
	lnC	lnBWCN	lnBWML
Fasting body weight	244 ±9	241 ±11	238 ±6
Pancreas	0.98 ±0.03	1.08 ±0.03	0.94 ±0.03
Liver	7.95 ±0.35	7.97 ±0.37	7.71 ±0.23
Gastrocnemius muscle	3.16 ±0.18	3.18 ±0.21	3.23 ±0.16
Peri-renal fat pad	1.67 ±0.22	1.66 ±0.14	1.50 ±0.22
Epididymal fat	2.24 ±0.20	2.15 ±0.13	1.95 ±0.21
Kidneys	2.12 ±0.14	2.19 ±0.07	2.15 ±0.12
Heart	0.99 ±0.06	0.88 ±0.03	0.93 ±0.02
Small intestine	4.57 ±0.20	4.36 ±0.17	4.60 ±0.12
Small intestine length (cm)	31.1 ±1.35	31.2 ±1.42	30.4 ±1.35

[1] Values provided are in grams except where indicated. Values are means ± SE. n=5/treatment group. There were no significant differences as determined by an ANOVA.

[2] lnC = lean rats fed control, lnBWCN = lean rats fed whole buckwheat concentrate supplement, lnBWML = lean rats fed milled buckwheat concentrate supplement.

Table C-13. Effect of dietary supplementation with a buckwheat concentrate for 3 weeks on urine and serum glucose of fa/fa Zucker rats.[1]

	Treatment Groups [2]		
	faC	faBWCN	faBWML
Urine glucose (mg/12 hours)	0.19 ±0.04[5]	0.19 ±0.02	0.19 ±0.05
Urine glucose (mg/mL)	0.03 ±0.01	0.04 ±0.01	0.03 ±0.01
Serum glucose[3] (mmol/L)	6.66 ±0.30[5]	7.76 ±0.14[6]	7.01 ±0.42[5]
Serum glucose[4] (mmol/L)	13.9 ±0.72	16.8 ±0.98	14.6 ±0.54
Urine D-*chiro*-inositol (nmol/12 hours)	72.3 ±7.0 [B]	859 ±156 [A]	980 ±198 [A]
Urine *myo*-inositol (nmol/12 hours)	372 ± 57	412 ± 49	492 ± 108

[1] Values are means ± SE. n=4/treatment group unless otherwise indicated. Means with different superscript letters indicate significant differences as determined by Duncan's multiple range test.
[2] faC = fa/fa rats fed control diet, faBWCN = fa/fa rats fed whole buckwheat concentrate supplement, faBWML = fa/fa rats fed milled buckwheat concentrate supplement.
[3] Serum was obtained from the saphenous vein prior to termination.
[4] Serum was obtained from the trunk blood collected following termination by CO_2 asphixyation and decapitation.
[5] n = 3 due to insufficient amount of serum obtained for analysis.
[6] n = 2 due to insufficient amount of serum obtained for analysis.

Table C-14. Effect of dietary supplementation with a buckwheat concentrate for 3 weeks on urine and serum glucose of lean Zucker rats.[1]

	Treatment Groups [2]		
	lnC	lnBWCN	lnBWML
Urine glucose (mg/12 hours)	0.17 ±0.04	0.17 ±0.05	0.21 ±0.04
Urine glucose (mmol/L)	0.05 ±0.02	0.06 ±0.02	0.05 ±0.02
Serum glucose[3] (mmol/L)	4.33 ±0.36	4.82 ±0.34	4.24 ±0.16
Urine D-*chiro*-inositol (nmol/12 hours)	78.6 ±24.3 ^{B}	321 ±70 ^{A}	350 ±114 ^{A}
Urine *myo*-inositol (nmol/12 hours)	314 ±49	232 ±50	210 ±46

[1] Values are means ± SE. n=5/treatment group. Means with different superscript letters indicate significant differences as determined by Duncan's multiple range test.

[2] lnC = lean rats fed control, lnBWCN = lean rats fed whole buckwheat concentrate supplement, lnBWML = lean rats fed milled buckwheat concentrate supplement.

[3] Serum was obtained from the trunk blood collected following termination by CO_2 asphixyation and decapitation.

Table C-15. Serum glucose concentrations during an oral glucose tolerance test of fa/fa Zucker rats after 2.5 weeks of dietary intervention with a buckwheat concentrate.[1]

Time (minutes)	Treatment Groups [2]		
	faC	faBWCN	faBWML
0	7.56 ±0.97	8.28 ±1.00	8.65 ±1.46
15	15.6 ±0.78	16.4 ±2.0	19.4 ±1.23[3]
30	14.0 ±0.8[3]	15.5 ±1.1	17.1 ±1.7[3]
60	7.97 ±0.41	10.1 ±2.2	13.7 ±3.5
90	5.36 ±1.83[4]	9.61 ±1.07[3]	9.94 ±0.79

[1] Values are expressed in mmol/L and are means ± SE. n=4/treatment group unless otherwise indicated. There were no significant differences between means at each time point as determined by an ANOVA.
[2] faC = fa/fa rats fed control diet, faBWCN = fa/fa rats fed whole buckwheat concentrate supplement, faBWML = fa/fa rats fed milled buckwheat concentrate supplement.
[3] n = 3 due to insufficient amount of serum obtained for analysis.
[4] n = 2 due to insufficient amount of serum obtained for analysis.

Table C-16. Serum glucose concentrations during an oral glucose tolerance test of lean Zucker rats after 2.5 weeks of dietary intervention with a buckwheat concentrate.[1]

Time (minutes)	Treatment Groups [2]		
	lnC	lnBWCN	lnBWML
0	7.34 ±0.47	7.42 ±0.21[3]	7.26 ±0.23
15	9.55 ±0.27	10.5 ±0.9[4]	8.84 ±2.97[5]
30	8.70 ±0.37[3]	8.95 ±0.65	8.47 ±0.55[3]
60	7.20 ±2.41[5]	7.02 ±2.38[5]	7.35 ±0.37
90	6.50 ±0.46	6.98 ±0.29[4]	7.04 ±0.46[3]

[1] Values are expressed in mmol/L and are means ± SE. n=5/treatment group unless otherwise indicated. There were no significant differences between means at each time point as determined by an ANOVA.
[2] lnC = lean rats fed control, lnBWCN = lean rats fed whole buckwheat concentrate supplement, lnBWML = lean rats fed milled buckwheat concentrate supplement.
[3] n = 4 due to insufficient amount of serum obtained for analysis.
[4] n = 3 due to insufficient amount of serum obtained for analysis.
[4] n = 2 due to insufficient amount of serum obtained for analysis.

Table C-17. Serum insulin concentrations during an oral glucose tolerance test of fa/fa Zucker rats after 2.5 weeks of dietary intervention with a buckwheat concentrate.[1]

Time (minutes)	Treatment Groups [2]		
	faC	faBWCN	faBWML
0	8.91 ±0.80	9.44 ±2.07	7.04 ±1.45
15	17.0 ±1.2	21.8 ±11.2[3]	13.1 ±2.2
30	18.4 ±2.0	19.1 ±3.7	20.6 ±4.8[3]
60	17.8 ±1.2	15.3 ±1.1	15.5 ±2.0[3]
90	18.3 ±0.6[4]	16.2 ±1.2[3]	18.0 ±0.8

[1] Values are expressed in ng/mL and are means ± SE. n=4/treatment group unless otherwise indicated. There were no significant differences between means at each time point as determined by an ANOVA.
[2] faC = fa/fa rats fed control diet, faBWCN = fa/fa rats fed whole buckwheat concentrate supplement, faBWML = fa/fa rats fed milled buckwheat concentrate supplement.
[3] n = 3 due to insufficient amount of serum obtained for analysis.
[4] n = 2 due to insufficient amount of serum obtained for analysis.

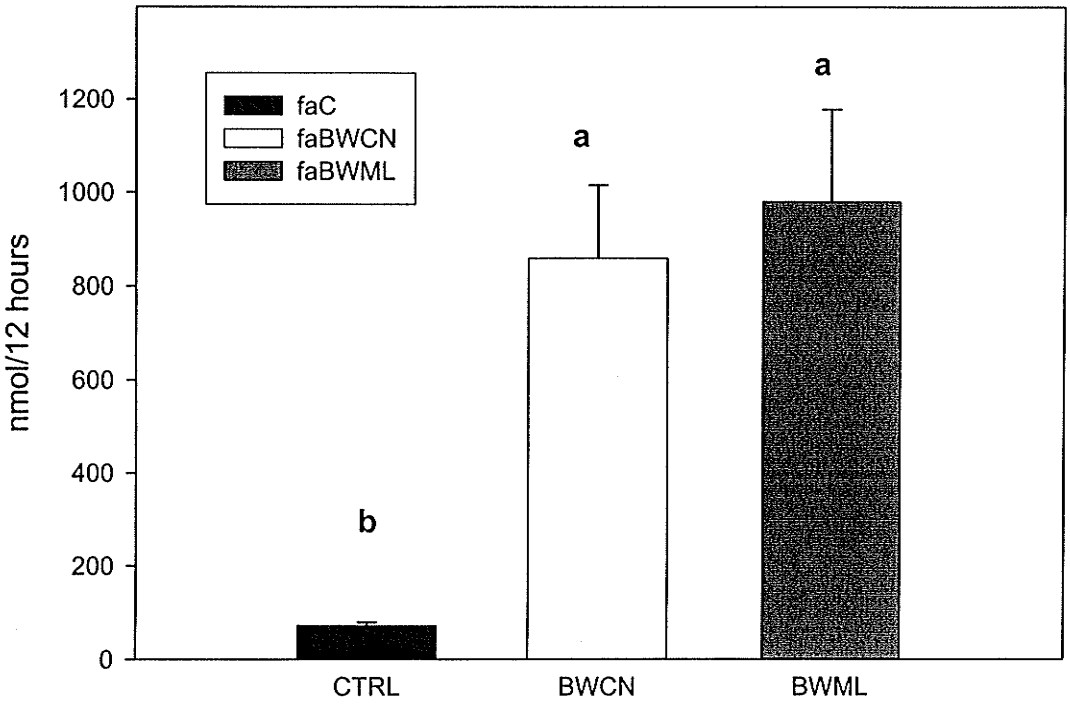


Figure C-1. Urinary D-*chiro*-inositol excretion in fa/fa Zucker rats following 3 weeks of dietary supplementation with buckwheat concentrates produced from either whole buckwheat (faBWCN) or milled buckwheat (faBWML) versus the rats fed the control diet (faC). Bars represent means ± SE for n=4 samples. Different letters indicate significant differences between groups as determined by Duncan's multiple range test ($p<0.05$).

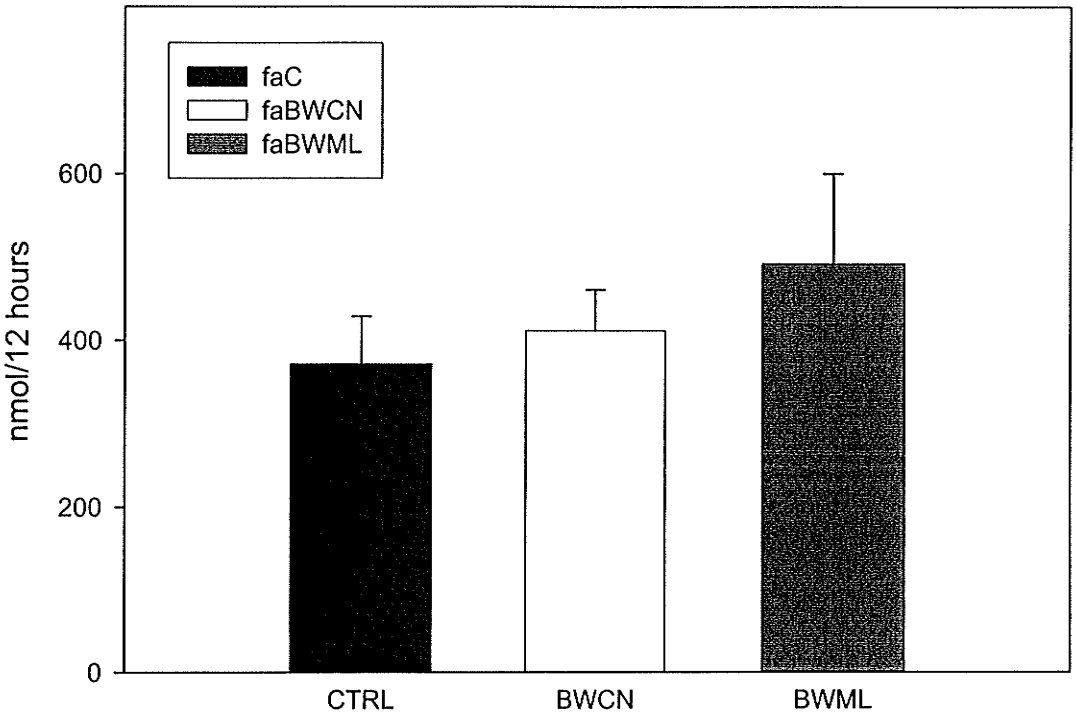


Figure C-2. Urinary *myo*-inositol excretion in fa/fa Zucker rats after 3 weeks of dietary supplementation with buckwheat concentrates produced from either whole buckwheat (faBWCN) or milled buckwheat (faBWML) versus the rats fed the control diet (faC). Bars represent means ± SE for n=4 samples. There were no significant differences between groups as determined by an ANOVA.

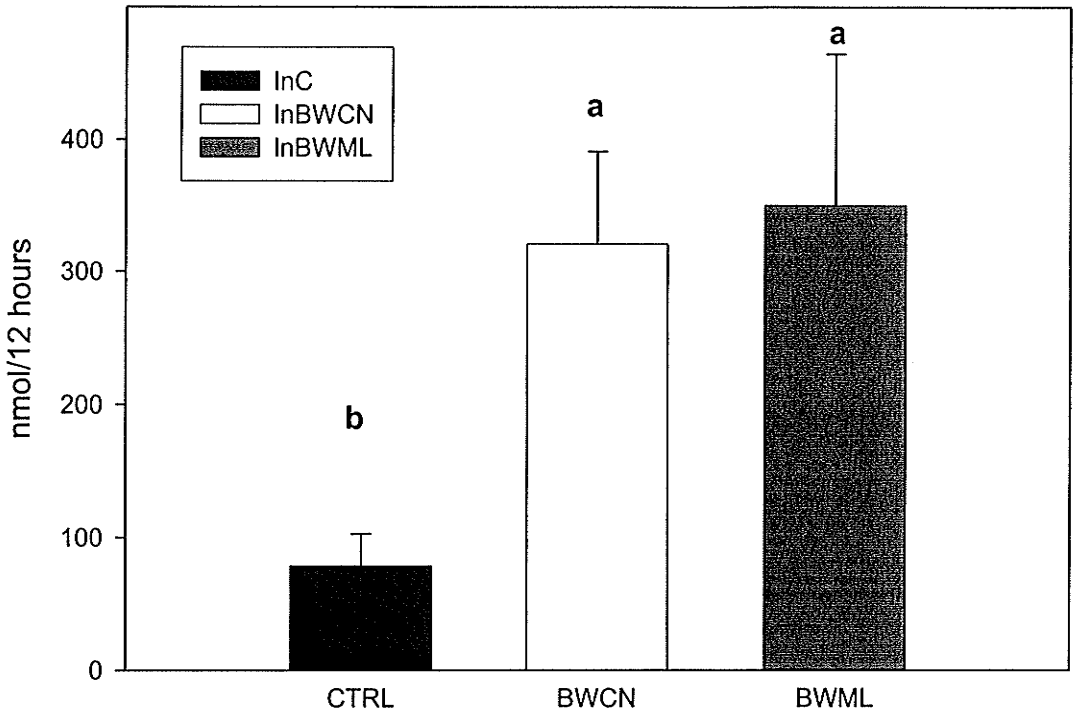


Figure C-3. Urinary D-*chiro*-inositol excretion in lean Zucker rats after 3 weeks of dietary supplementation with buckwheat concentrates produced from either whole buckwheat (InBWCN) or milled buckwheat (InBWML) versus the rats fed the control diet (InC). Bars represent means ± SE for n=5 samples. Different letters indicate significant differences between groups as determined by Duncan's multiple range test ($p<0.05$).

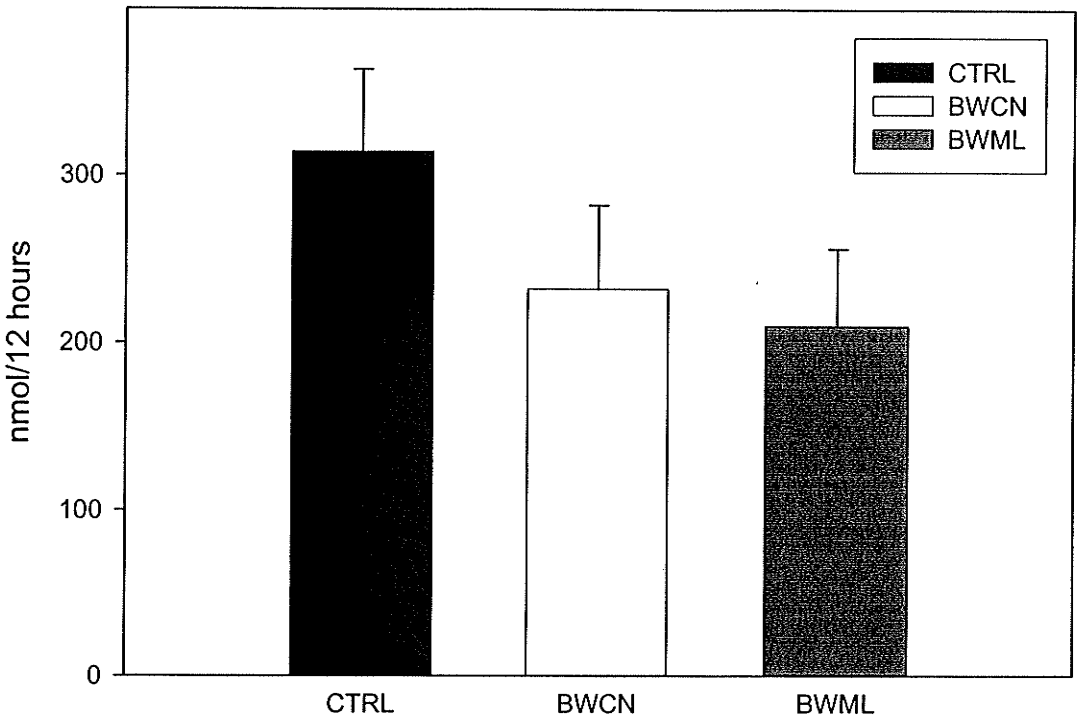


Figure C-4. Urinary *myo*-inositol excretion in lean Zucker rats after 3 weeks of dietary supplementation with with buckwheat concentrates produced from either whole buckwheat (lnBWCN) or milled buckwheat (lnBWML) versus the rats fed the control diet (lnC). Bars represent means ± SE for n=5 samples. There were no significant differences between groups as determined by an ANOVA.

Appendix D: Additional experiments evaluating the molecular and cellular mechanisms for the antihyperglycemic effects of buckwheat

Treatment of H4IIE cells with additional inhibitors

PLD and a member of the src family were required for insulin and buckwheat concentrate (BWC)-stimulated MAPK phosphorylation (Chapter 7). We also investigated the effects of other inhibitors on the ability of insulin and the buckwheat concentrate (BWC) to stimulate phosphorylation of MAPK, PDK-1 and Raf. Cell culture, treatment and inhibitor details are provided in Chapter 7. Results from these experiments are shown in Tables D-1, D-2 and D-3. As expected, insulin and BWC-stimulated phosphorylation of MAPK was inhibited by PD98059, whereas no other inhibitors had any effects. This indicates that G-protein, PI3K, Stat3, InsR, Raf, PLC, PKA and PKC are not required for MAPK phosphorylation by insulin or the BWC in H4IIE cells. In addition, phosphorylation of PDK-1 and Raf was not stimulated by either insulin or the BWC, and subsequently none of the inhibitors had any effects on these proteins.

Table D-1. The effects of inhibitors on insulin stimulated phosphorylation of selected signal transduction proteins.

Antibodies	Inhibitors							
	None	PTX	LY	PD	AG490	AG1024	Raf	U73
p42/44 P-MAPK (Thr^{202}/Tyr^{204})	13.3	11.2	10.4	1	16.4	16.74	17.7	13.4
PDK-1 (Ser^{241})	1.57	1.71	1.40	1.89	1.91	2.30	1.79	2.30
Raf (Ser^{259})	0.53	0.29	0.22	0.20	0.13	0.26	0.29	0.99

Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with insulin for 6 minutes with untreated cells serving as the control. Inhibitors of the phosphorylation of G-protein (pertussis toxin or PTX), PI3K (LY294002 or LY), MAPK (PD98059 or PD), Stat3 (AG490), InsR (AG1024), Raf (Raf kinase inhibitor or Raf), and PLC (U73122 or U73) were included. Further details on inhibitors and antibodies are provided in Chapter 6. Concentrates were subsequently prepared for Western blot analysis. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00 and data presented are from one experiment.

Table D-2. The effects of inhibitors on the buckwheat concentrate (BWC) stimulated phosphorylation of selected signal transduction proteins.

	Inhibitors						
Antibodies	None	PTX	LY	PD	AG490	Raf	U73
p42/44P-MAPK (Thr^{202}/Tyr^{204})	15.8	14.4	13.1	1.00	12.9	16.9	8.6
PDK-1 (Ser^{241})	0.82	0.58	0.61	0.54	0.89	0.27	0.49
Raf (Ser^{259})	0.30	0.28	0.08	0.20	0.16	0.12	0.12

Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with the BWC for 6 minutes with untreated cells serving as the control. Inhibitors of the phosphorylation of G-protein (pertussis toxin or PTX), PI3K (LY294002 or LY), MAPK (PD98059 or PD), Stat3 (AG490), Raf (Raf kinase inhibitor or Raf), and PLC (U73122 or U73) were included. Further details on inhibitors and antibodies are provided in Chapter 6. Concentrates were subsequently prepared for Western blot analysis. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00 and data presented are from one experiment.

Table D-3. The effects of inhibitors on phosphorylation of mitogen-activated protein kinase (MAPK) by insulin or the buckwheat concentrate (BWC).

Treatments	Inhibitors			
	None	Rottlerin	Go6976	Go7874
Insulin	3.53	2.46	4.68	2.60
BWC	5.27	3.85	4.32	3.10

Following a 15 minute preincubation with the indicated inhibitors, H4IIE cells were treated with insulin or the BWC for 6 minutes with untreated cells serving as the control. Inhibitors of PKC (Rottlerin) and PKA (Go6976 and Go7874) were included. Further details on inhibitors and treatments are provided in Chapter 6. Concentrates were subsequently prepared for Western blot analysis with p42/44 P-MAPK (Thr^{202}/Tyr^{204}) antibody. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00 and data presented are from one experiment.

Treatment of FAO cells

To ensure the effects of the buckwheat concentrate (BWC) in H4IIE cells were not unique to the cell type, we performed additional experiments using FAO cells. FAO cells are related to H4IIE since both cell lines were derived from Reuber H35 cells (Richardson *et al*, 1969). Likewise, they exhibit a number of hepatocyte-specific differentiation markers and remain sensitive to insulin (Snodgrass and Lin, 1987; Szanto and Kahn, 2000). FAO hepatoma were propogated under the same culture conditions employed for H4IIE hepatomas. Quiescent FAO cells were obtained by placing the cells into serum-free medium for 3 days. FAO cells were treated as previously described for H4IIE cells in Chapter 7 (Section 7.3).

Results from these experiments are presented in Table D-4 and Figures D-1, D-2 and D-3. As shown in Table D-4 and Figure D-1, insulin and the BWC stimulated phosphorylation of MAPK in FAO cells wheras D-*chiro*-inositol and *myo*-inositol did not. This is the same result as in H4IIE cells, indicating that the observations presented in Chapter 7 are not unique to the H4IIE cell type. Furthermore, the lack of insulin and BWC-stimulated phosphorylation of PDK-1 and Raf in H4IIE cells was also observed in FAO cells (Table D-4, Figure D-2 and D-3).

Table D-4. Stimulation of protein phosphorylation by insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) of select signal transduction proteins in FAO cells.

Antibody	Insulin	BWC	D-CI	MI
p42/44 MAPK	0.90	0.98	1.34	1.44
p42/44 P-MAPK (Thr^{202}/Tyr^{204})	29.3	5.56	1.74	0.76
Raf (Ser^{259})	0.81	0.55	0.77	0.85
PDK-1 (Ser^{241})	0.70	0.82	0.80	0.71

FAO cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control. Stimulation of proteins in cellular concentrates was determined by Western blotting. Treatment and antibody specifications are provided in Chapter 6. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00. Data presented is from one experiment.

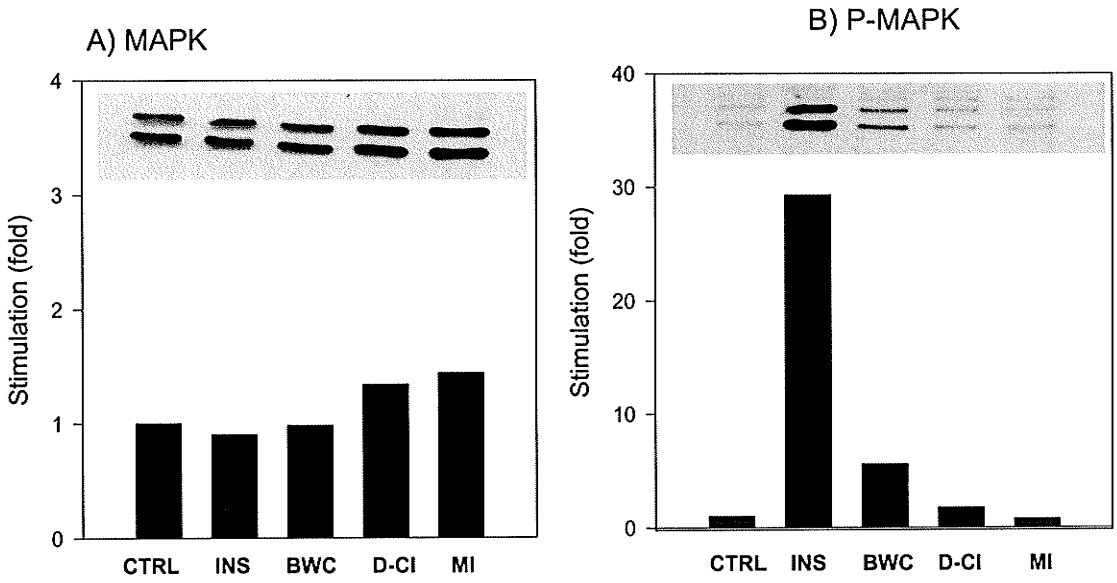


Figure D-1. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of mitogen-activated protein kinase (MapK) in FAO cells. Cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Band intensities on each blot were quantified by scanning densitometry. This figure illustrates data from one experiment.

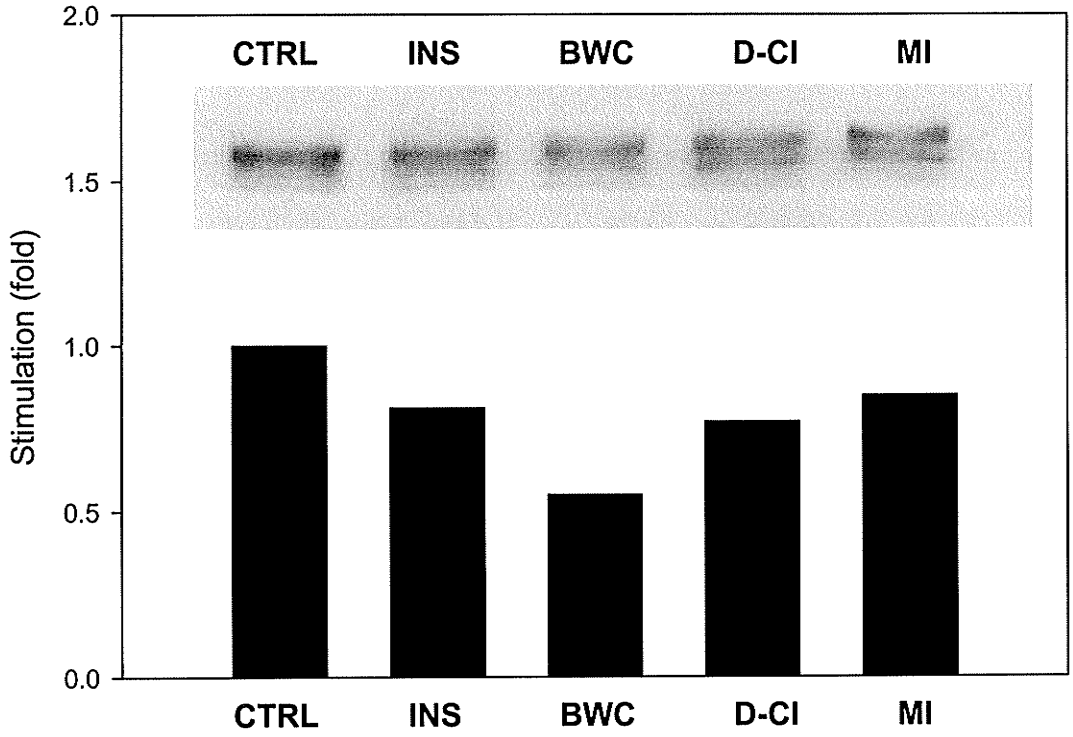


Figure D-2. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of Raf in FAO cells. Cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Band intensities on each blot were quantified by scanning densitometry. This figure illustrates data from one experiment.

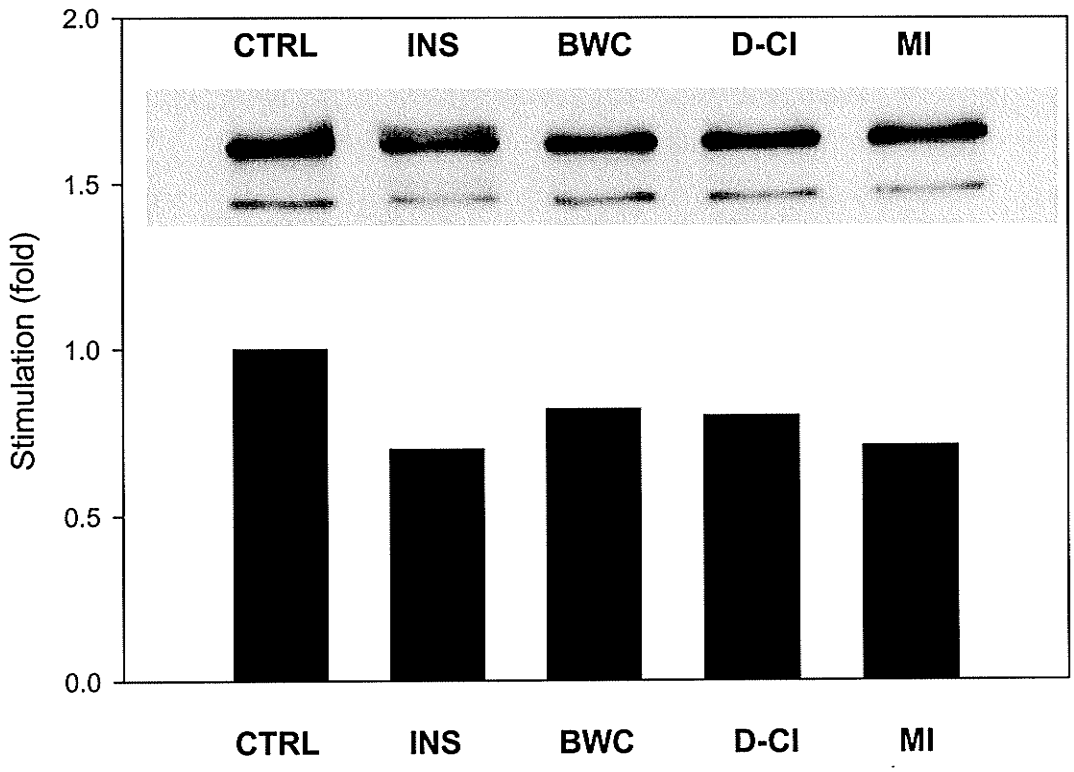


Figure D-3. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI) and *myo*-inositol (MI) on phosphorylation of PDK1 in FAO cells. Cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Band intensities on each blot were quantified by scanning densitometry. This figure illustrates data from one experiment.

Treatment of H4IIE cells with carbohydrate standards

To determine the activity of individual components present in the buckwheat concentrate, we tested the effects of chemical standards on signaling proteins with demonstrated responsiveness to the buckwheat concentrate (BWC). Standards were dissolved in water at an equal concentration to the amounts present in the BWC (Appendix B, Table B-17) and 2 μL of each solution (0.1% v/v final concentration) was added directly to cells. As shown in Table D-5 and Figure D-6, both insulin and the BWC stimulated phosphorylation of MAPK whereas none of the carbohydrate standards present in the BWC were effective for stimulating MAPK phosphorylation.

Table D-5. The effects of insulin (INS), the buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI), *myo*-inositol (MI) and standards of carbohydrates present in the BWC on mitogen activated protein kinase phosphorylation.

Antibody	Treatments									
	INS	BWC	FR	GL	D-CI	MI	SUC	RF	ST	Veh
MAPK	0.70	0.71	0.84	0.70	0.85	0.74	0.73	0.63	0.71	0.89
P-MAPK	598	520	7.00	1.50	3.00	0.00	0.00	1.00	2.50	2.00
RATIO	851	732	8.34	2.14	3.55	0.00	0.00	1.59	3.53	2.26

Treatments of soluble carbohydrate standards included fructose (FR), glucose (GL), sucrose (SUC), raffinose (RF), stachyose (ST), and the vehicle (Veh) and were added to cells in the equivalent amount to that present in the BWC. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control. Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) were determined by Western blotting. The ratio of P-MAPK/MAPK is also presented (RATIO). Treatment and antibody specifications are provided in Chapter 6 and Appendix A. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00. Data presented is from one experiment.

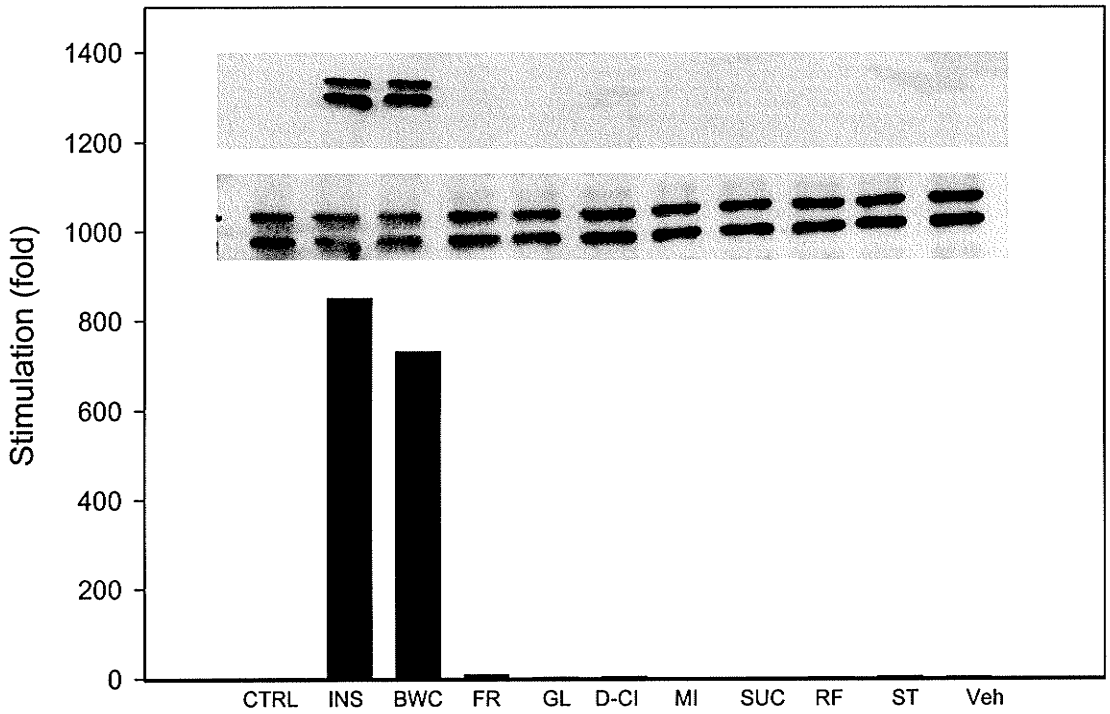


Figure D-6. The effects of insulin (INS), buckwheat concentrate (BWC), D-*chiro*-inositol (D-CI), *myo*-inositol (MI) and carbohydrate standards on mitogen-activated protein kinase (MAPK). Treatments of soluble carbohydrate standards included fructose (FR), glucose (GL), sucrose (SUC), raffinose (RF), stachyose (ST), and the vehicle (Veh) and were added to cells in the equivalent amount to that present in the BWC. H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control (CTRL). Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) in cellular protein concentrates were determined by Western blotting. Band intensities on each blot were quantified by scanning densitometry. Results are presented as the ratio of P-MAPK/MAPK in stimulation (fold) versus the CTRL. The value for CTRL is 1.00. This figure illustrates data from one experiment.

Treatment of H4IIE cells after centrifugal separation of the buckwheat concentrate

The consistency of the buckwheat concentrate (BWC) was such that particles would precipitate out of solution when the concentrate was left to sit. For all cell culture experiments, the BWC was mixed prior to its addition to cells, however, we also evaluated the effects of centrifugal separation of these larger molecules from those remaining in solution on stimulation of signal transduction proteins in H4IIE cells. As shown in Table D-7, the supernatant, pellet and EtOH were not effective for stimulating MAPK phosphorylation compared to the whole BWC, indicating the requirement for uniform mixing of the BWC prior to its addition to cells.

Table D-7. The effects of the buckwheat concentrate (BWC) before and after centrifugation.

Antibody	BWC	supernatant	pellet	EtOH
MAPK	0.79 ± 0.05	1.07 ± 0.20	0.82 ± 0.08	0.80 ± 0.21
P-MAPK	1.88 ± 0.48	1.11 ± 0.09	1.05 ± 0.02	0.98 ± 0.03
RATIO	2.42 ± 0.76	1.06 ± 0.11	1.29 ± 0.09	1.32 ± 0.37

H4IIE cells were treated with each agent individually for 6 minutes, with untreated cells serving as the control. Treatments included the BWC prior to centrifugation and the supernatant and pellet collected following centrifugation. Ethanol (EtOH) was added to cells in an amount equivalent to that of the BWC to account for the potential presence of EtOH in the BWC. Each experiment was replicated three times. Stimulation of p42/44 MAPK and p42/44 phosphorylated (P) MAPK (Thr^{202}/Tyr^{204}) were determined by Western blotting. The ratio of P-MAPK/MAPK is also presented (RATIO). Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00 ± 0.00 and all data are expressed as means ± SE (n=3).

Fractionation of the buckwheat concentrate

The following method was developed to separate and collect fractions from the buckwheat concentrate (BWC). The objective of this experiment was to obtain fractions containing isolated components (D-*chiro*-inositol, *myo*-inositol, sucrose, and fagopyritols) from the BWC that could be tested for their ability to stimulate signal transduction proteins in H4IIE cells. We evaluated the effects of isolated fractions on stimulation of MAPK phosphorylation as compared to insulin and the whole BWC. As shown in Table D-8, the fractions were generally less effective than the whole BWC and insulin for MAPK phosphorylation, however, further investigation using more concentrated fractions may reveal certain fractions that have high bioactivity in terms of MAPK phosphorylation.

a) Reagents

- Buffer (25 mM NaCl and 20 mM (pH 7.4)Tris•HCl)
- Bio-Gel P2 Gel Fine (45-90 μM wet) salts (Bio-Rad)

b) Procedure

BioGel P2 was hydrated in deionized water overnight. Excess liquid was removed. A column of Bio-Gel P2 was prepared by slowly pouring the slurry into a 1 cm diameter glass column one-third full of water. Once the Bio-Gel had settled, the spigot was opened and the liquid allowed to pack the gel (1.0 cm x 19 cm). The column was subsequently washed with 2 column volumes of the wash buffer prior to loading the sample. The BWC (680 μL) was gently vortexed and was placed onto the column and allowed to pass into the gel before elution with wash buffer. Fractions of 26 drops (~500 μL) were collected and MAPK

phosphorylation activity was detected by Western blot analysis after treating H4IIE cells with 25 μL of each fraction. Cells were cultured and treated as described in Chapter 7 except that cells were in 24 well plates (containing 1 mL media/well) and 1 μL of the BWC was added to cells instead of 2 μL.

Table D-8. The effects of insulin (INS), the buckwheat concentrate (BWC), and fractions isolated from the BWC on phosphorylation of p42/44 MAPK (Thr^{202}/Tyr^{204}) as determined by Western blotting.

Blot A		Blot B	
Fraction	P-MAPK	Fraction	P-MAPK
INS	16.4	INS	15.0
BWC	4.00	BWC	7.95
6	2.33	13	0.47
7	0.67	14	1.03
8	3.33	15	0.76
9	0.33	16	0.79
10	1.22	17	0.63
11	0.44	18	0.45
12	0.33	19	0.68
		20	0.61
		21	0.82
		22	2.29
Blot C		Blot D	
Fraction	P-MAPK	Fraction	P-MAPK
INS	1.90	INS	6.46
BWC	3.43	BWC	12.85
23	0.14	30	0.92
24	0.29	31	1.38
25	0.38	32	0.54
26	0.33	33	0.54
27	0.67	34	0.38
28	0.48	35	1.15
29	0.33	36	0.46
		37	1.31
		38	0.62
		39	0.85
		40	1.08

H4IIE cells in 24-well plates were treated with either 25 μL of fractions, 1 μL of the BWC, or 250 nM insulin for 6 minutes, with untreated cells serving as the control. Stimulation of P-MAPK was determined by Western blotting. In Table D-8, Blots A, B, C, and D represent different Western blots. Band intensities on each blot were quantified by scanning densitometry and are presented as stimulation (fold) relative to the control. The values for the control are 1.00. Data presented is from one experiment.

Microarray analysis of RNA isolated from buckwheat-treated rats

As shown in Chapter 6, *in vivo* administration of the buckwheat concentrate (BWC) lowered serum glucose concentrations in streptozotocin (STZ) rats. Using the same animal model and test conditions established as having a positive response to the BWC, we investigated the *ex-vivo* molecular changes related to glucose metabolism and insulin signaling using a microarray analysis. The following sections provide a description of the methods used to obtain the RNA samples from buckwheat-treated rats. Results of the microarray analysis are provided in Tables found on the CD insert on page D-28.

Animals and treatments

STZ rats in the fed state were given an acute dose of a BWC (20 mg D-*chiro*-inositol/kg body weight) or a placebo (n=4/group) as described in Chapter 6. Ninety minutes following administration of the BWC, animals were terminated by CO_2 asphyxiation and cervical dislocation following Canadian Council on Animal Care Guidelines. The livers were excised and immediately frozen in liquid nitrogen. All tissue samples and serum were stored at -80°C until required for RNA isolation.

Extraction of total RNA

TRIzol is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent is a monophasic solution of phenol and guanidine isothiocyanate.

a) Reagents:

- TRIzol (Invitrogen)
- Nuclease-free H_2O (Invitrogen)
- General laboratory chemicals were purchased from Fisher and Sigma.

b) Procedure:

Homogenization

While immersed in liquid nitrogen, 50 mg of liver tissue was crushed into a fine powder with a mortar and pestle. TRIzol (1 mL) was added to the mortar, mixed with the dried sample and transferred to a 15 mL conical tube. The sample was homogenized for 10-15 seconds, incubated for 5 minutes at room temperature, and transferred to a 1.5 mL microfuge tube.

Phase separation

Chloroform (200 μL) was added to the sample, shaken vigorously by hand for 15 seconds, and incubated at room temperature for 2 – 3 minutes. Samples were centrifuged (Beckman Microfuge 18) at 12 000 rpm for 15 minutes at 4°C.

RNA precipitation

Approximately 500 μL of the upper aqueous phase was transferred to a new microfuge tube, then 500 μL ice-cold iso-propanol was added and the tube was gently inverted several times. Samples were incubated for 10 minutes at

room temperature and gently mixed every 5 minutes during incubation. Samples were centrifuged (Beckman Microfuge 18) at 10 000 x g for 15 minutes at 4°C.

RNA Wash

The supernatant was removed from samples and the remaining RNA pellet washed with 1 mL of ice-cold 70% ethanol. Samples were gently vortexed and centrifuged (Beckman Microfuge 18) at 10 000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed with 1 mL of ice-cold 70% ethanol. The supernatant was discarded and the microfuge tube was carefully dried with a Kim-Wipe tissue without disturbing the pellet. Tubes were inverted and left to dry for 10 minutes.

Redissolving the RNA

To each tube, 15 μL of nuclease-free H_2O was added. Tubes were incubated in a 50°C water bath for 10 minutes and RNA was stored at -80°C.

Measurement of RNA concentration

The individual nucleotide bases that constitute the RNA molecule absorb light in the ultraviolet region of the electromagnetic spectrum. The absorbance of these moieties is related to the concentration of RNA in solution through the Beer-Lambert Law. The Beer-Lambert law states that the absorbance of a compound at a specific wavelength is directly proportional to the concentration of that compound in solution. Empirically, an optical density measurement of 1.0 at a wavelength of 260 nm corresponds to approximately 40 μg/mL of RNA.

Procedure

Aliquots of each RNA sample (4.4 μL) were placed into 1.5 mL microfuge tubes and 996 μL of deionized water was added to each tube. To determine the RNA concentration, the absorbance of samples and a standard (deionized water only) was determined spectrophotometrically at 260 nm. To determine the purity of RNA samples, the absorbance of samples and a standard (deionized water only) was determined at 280 nm.

Calculations

The concentration of RNA (μg/μL) = Absorbance at 260 nm x 10

The purity of RNA samples was based on the ratio of Absorbance at 260 nm/Absorbance at 280 nm. A pure sample of RNA will have an OD_{260}/ OD_{280} of 2.0 ± 0.15.

Table D-9. Concentrations and purity of RNA isolated from STZ rat liver.

Treatment	Rat #	µg/uL	OD_{260}/ OD_{280} ratio
Buckwheat	1	1.36	1.79
Buckwheat	2	0.5	2.17
Buckwheat	3	0.88	1.91
Buckwheat	4	1.11	1.73
Placebo	1	1.06	1.8
Placebo	2	0.66	2
Placebo	3	0.86	1.56
Placebo	4	0.47	1.74

Agarose gel electrophoresis of RNA

a) Reagents:

- agarose LE for RNA
- 50 x Tris acetate EDTA (TAE) Running buffer:

  2.0 M Tris, 5.7% glaD-ClaI acetic aD-CId, 0.05 M EDTA (pH 8.0)
- 1% ethidium bromide (w/v)
- Nuclease free H_2O
- 4 x RNA loading buffer:

  67% formamide, 21% formaldehyde, 0.03 M MOPS (pH 7.4), 1.5 mM EDTA, 0.1% SDS, 6.6% glycerol, 0.1% bromophenol blue/xylene cyanol FF, 0.5% DEPC-treated purified H_2O.

b) Procedure

Preparation of agarose gel

A 1% (w/v) agarose solution was prepared using 1 x TAE buffer (prepared from 50 x TAE buffer). The 1% agarose solution was heated until the agarose melted. The electrophoresis tray was assembled with gel gates and the comb was placed in the tray so that the minimum distance between the comb and tray was 1 mm. Once the agarose solution was cooled to lukewarm (comfortably held on hand), it was used to seal the space between the gates and tray and then poured into the tray on a level surface and allowed to solidify at 4°C for approximately 10 minutes or at 18-22°C for 30 minutes. At room temperature, the running buffer was added to the tray to a depth of approximately 2-3 mm above the agarose gel. The comb and gel gates were carefully removed from the gel. The gel tray

was transferred to the electrophoresis chamber such that the wells were closer to the negative terminal of the gel box.

Sample preparation

Samples were prepared on ice. One μL of each sample was combined with 8 μL of nuclease free H_2O, 3 μL of 4 x RNA loading buffer and 0.1 μL of ethidium bromide stock. Twelve μL of prepared samples were then loaded onto the gel.

Running the gel

The cover of the electrophoretic chamber was closed and connected to the power supply. The gel was run at 50 mA for 30 minutes. The separation was viewed under UV light and documented by photography.

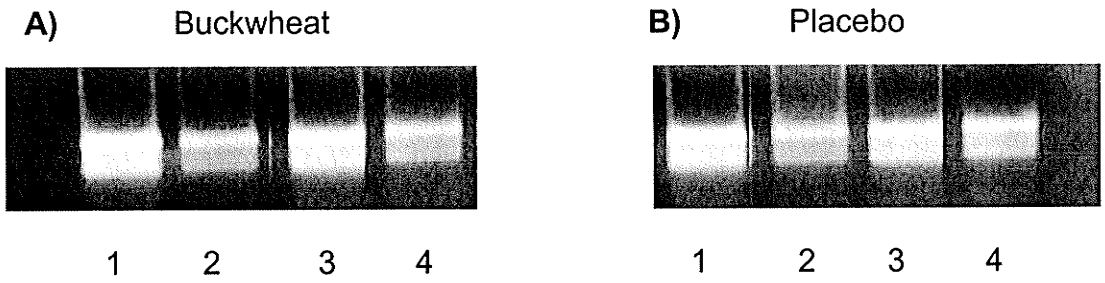


Figure D-4. Separation of RNA isolated from STZ rat livers. STZ rats were given an acute dose of A) a buckwheat concentrate or B) placebo. The RNA samples were pooled for the microarray analysis by combining 5 μg RNA from 4 individual samples in each treatment group.

Microarray Analysis

The microarray analysis of liver RNA isolated from buckwheat treated STZ rats versus rats given a placebo was conducted by Med BioGene (Vancouver, BC). The raw data from the buckwheat-treated rats were normalized versus the placebo group to identify differentially expressed genes. Changes greater or less than 3-fold are considered significant.

Results

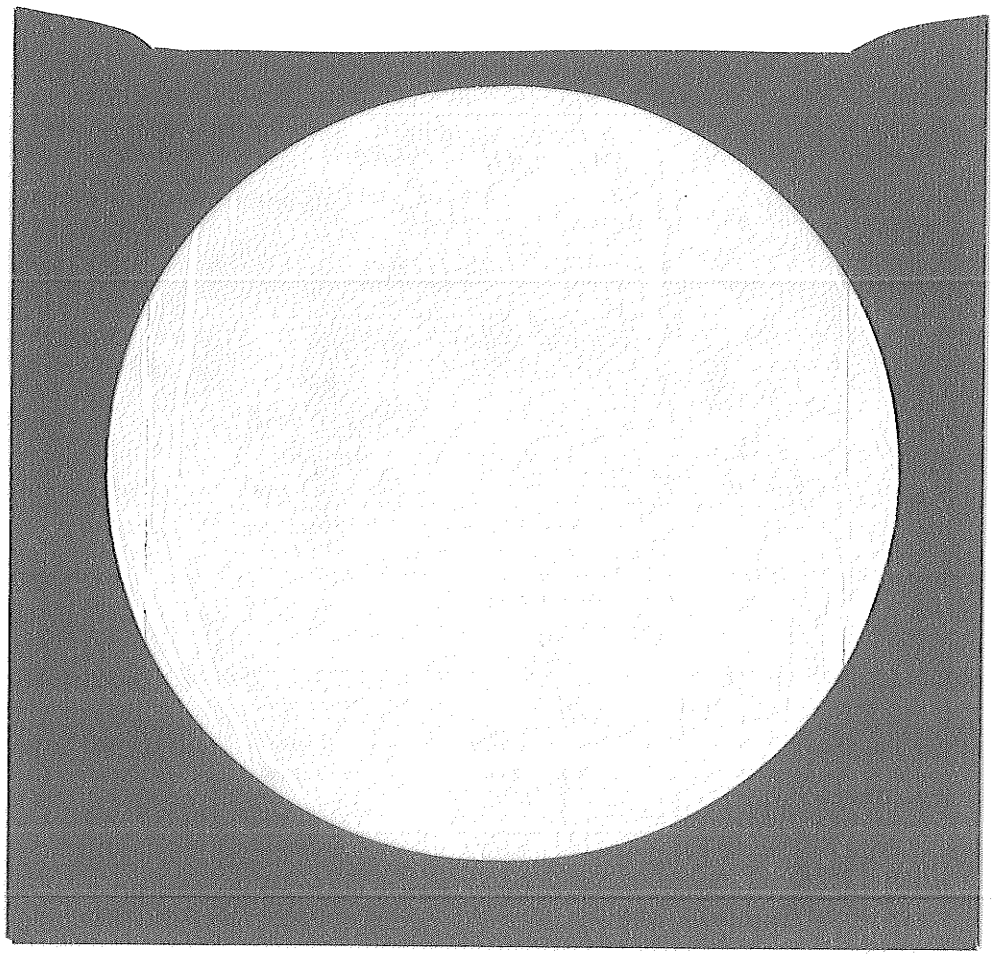
See CD insert

Table D-10. Results from microarray analysis of liver RNA from placebo-treated rats

Table D-11. Results from microarray analysis of liver RNA from buckwheat-treated rats

Table D-12. Genes upregulated by buckwheat

Table D-13. Genes downregulated by buckwheat



Measurement of PLD activity in H4IIE cells

The buckwheat concentrate (BWC) required PLD for stimulation of MAPK phosphorylation, as described in Chapter 7. We also investigated the effects of the BWC on PLD activity in H4IIE cells. The method employed is described below. Our results suggest that the BWC does not stimulate PLD activity in H4IIE cells (data not shown).

Materials and reagents

- HEPES-Tyrode buffer (129 mM NaCl, 8.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM KH_2PO_4, 56 mM glucose, 10 mM Hepes (pH 7.4), 0.8 mM $MgCl_2$)
- ^{3}H-palmitic aD-Cld was purchased from Perkin-Elmer.

Cell culture

Rat H4IIE hepatoma cells were cultured as described in Chapter 7.

Assay of PLD activation in H4IIE cells

Determination of PLD activity was performed according to a modified method of Banno *et al* (1999). Quiescent H4IIE cells in 12 well plates containing 2 mL media/well were labeled overnight with 2.5 μD-Cl/mL ^{3}H-palmitic aD-Cld. Cells were washed with SMC-PBS (0.9% NaCl, 0.1 M Na_3PO_4, pH 7.1) and preincubated in HEPES-Tyrode buffer containing 0.3% 1-butanol (v/v) (2 mL/well) ± inhibitors for 10 minutes. Following the preincubation, cells were stimulated with treatments for 30 minutes. The reactions were terminated by removing the media followed by the immediate addition of ice-cold H_2O:methanol (2:5, v/v) (1 mL/well). Lipids were then concentrated according to the method of Folch *et al* (1957). Briefly, cellular concentrates were removed from tissue

culture plates and placed into plastic tubes. Chloroform and water were added to the samples to achieve a ratio of 0.8:0.4:0.3 (v/v/v) for chloroform, methanol, and water, respectively. Samples were mixed on a vortex and allowed to clarify into 2 layers. The upper alcohol layer was removed and the lower chloroform layer, containing the lipids was dried in a speed-vac. The dried lipids were resuspended in small volume of chloroform and separated on a Silica Gel 60 plate by one-dimensional thin-layer chromatography in a solvent system using an upper phase of ethyl acetate/2,24-trimethylpentane/acetic acid/water (13:2:3:10, v/v/v/v).