Université de Montréal

Antidiabetic activity of *Vaccinium vitis-idaea*, a medicinal plant from the traditional pharmacopeia of the James Bay Cree

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Cette thèse intitulée:

Antidiabetic activity of *Vaccinium vitis-idaea*, a medicinal plant from the traditional pharmacopeia of the James Bay Cree

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Résumé

L'incidence du diabète chez les premières nations du Canada est plus de trois fois celle du reste du pays, dû, en partie, aux traitements culturellement inappropriés. Notre projet vise à traiter le diabète chez ces populations à partir de leur pharmacopée de médicine traditionnelle afin d'améliorer l'acceptation des traitements. En utilisant une approche ethnobotanique, notre équipe a identifié 17 plantes médicinales utilisées pour traiter des symptômes du diabète par les Cris d'Eeyou Istchee (Baie James, Québec). Parmi eux, l'extrait éthanolique de baies de *Vaccinium vitis-idaea* a montré un effet stimulateur sur le transport du glucose dans les cellules musculaires squelettiques et les adipocytes en culture. Le but de cette thèse était d'élucider les mécanismes par lesquels cet extrait exerce ses effets anti-hyperglycémiants, d'identifier ses principes actifs et de confirmer *in vivo*, son efficacité. Les résultats démontrent que *V.vitis* a augmenté le transport du glucose dans les cellules musculaires en cultures, C2C12 et L6 et a stimulé la translocation des transporteurs GLUT4 dans les cellules L6. L'extrait a également inhibé la respiration dans les mitochondries isolées du foie du rat. Cet effet est semblable à celui de la metformine et en lien avec la production du stress métabolique et l'activation de l'AMPK. De plus, la voie de signalisation de l'insuline ne semble pas être impliquée dans le mécanisme d'action de *V. vitis*.

Le fractionnement guidé par la stimulation du transport du glucose a mené à l'isolation des principes actifs; la quercétine, la quercétine-3-*O*-galactoside, et la quercétine-3-*O*-glucoside. Comparable à l'extrait brut, ses composés ont stimulé la voie AMPK. Cependant, la quércetine était la seule à inhiber la respiration mitochondriale.

Pour valider l'effet de *V.vitis in vivo*, l'extrait (1% dans l'eau de boisson) a été administré aux souris KKA^y pendant 10 jours. La glycémie et le poids corporel ont été significativement réduits par *V.vitis*. Ces effets ont été associés à une diminution de la prise alimentaire, ce qui suggère que *V.vitis* diminue l'appétit. L'étude pair-fed a confirmé que les effets de *V.vitis* sont, majoritairement, dû à la réduction de l'appétit. De plus, *V.vitis* a augmenté la teneur en GLUT4 dans le muscle squelettique, a stimulé la

phosphorylation de l'ACC et a augmenté les niveaux de PPAR-α dans le foie des souris KKA^y. Ces effets se voient être additifs à l'effet anorexigène de *V. vitis*.

Au cours du fractionnement bioguidé de l'extrait, l'ester méthylique de l'acide caféique (CAME), un produit formé lors de la procédure du fractionnement, a démontré un effet stimulateur puissant sur le transport du glucose dans les celules C2C12 et donc un potentiel anti-diabétique. Pour identifier d'autres acides caféique active (AC) et pour élucider leurs relations structure-activité et structure-toxicité, vingt dérivés AC ont été testés. Outre CAME, quatre composés ont stimulé le transport du glucose et ont activé l'AMPK suite au stress métabolique résultant d'un découplage de la phosphorylation oxydative mitochondriale. L'activité nécessite une fonction d'AC intacte dépourvu de groupements fortement ionisés et ceci était bien corrélée avec la lipophilicite et la toxicité. Les résultats de cette thèse soutiennent le potentiel thérapeutique de *V. vitis*, ses composés actifs ainsi que de la famille de l'AC et pour la prévention et le traitement du diabète.

Mots-clés : *Vaccinium vitis*, diabète de type 2, AMPK, ACC, PPAR-α, OPD, KKA^y, GLUT4, produits de santé naturels, médecine traditionnelle, la forêt boréale canadienne, les Autochtones d'Amérique du nord.

Abstract

Type 2 diabetes in Canadian First Nations is three times higher than the national average. Poor prognosis is partly attributed to cultural inappropriateness of pharmaceutical products. Our project aims to develop culturally adapted diabetes treatment based on traditional medicine pharmacopoeia. Our team has identified 17 plants used to treat the symptoms of diabetes by the Cree of Eeyou Istchee (James Bay, Quebec). Among them, the ethanol extract of *Vaccinium vitis-idaea* berries was found to have an important stimulatory effect on glucose uptake in cultured skeletal muscle cells and adipocytes. The goal of this thesis was to elucidate the mechanisms of action of this plant product as well as to isolate and identify its active constituents using a bioassay-guided fractionation approach and finally to validate the antidiabetic activity *in vivo*. The extract of *V.vitis* enhanced glucose uptake in cultured C2C12 and L6 skeletal muscle cells and stimulated the translocation of GLUT4 transporters to the cell membrane of L6 cells. It mildly inhibited ADP-stimulated oxygen consumption in isolated rat liver mitochondria, an effect similar to that of metformin and consistent with metabolic stress and the consecutive activation of AMP-activated protein kinase (AMPK) pathway. The insulin pathway does not seem to be involved in *V.vitis* signaling.

Fractionation of this plant extract, guided by glucose uptake activity, resulted in the isolation of the active principles, quercetin-3-*O*-galactoside, quercetin, and quercetin-3-*O*-glucoside. Similar to the crude extract, the quercetin glycosides and the aglycone stimulated the AMPK pathway. However, only the aglycone inhibited ATP synthase in isolated mitochondria.

To validate the effect of *V.vitis in vivo*, the extract (1% in drinking water) was administered to KKA^y mice for 10 days. Glycemia and body weight were significantly reduced by *V.vitis*. These effects were associated with decrease of food intake, suggesting that *V.vitis* reduces the appetite. The pair-fed study confirmed that the previous effects of *V.vitis* are almost mediated by its appetite reducing action. In addition, *V. vitis*-treatment increased the content of GLUT4 protein in skeletal muscle, stimulated the phosphorylation of ACC and increased the levels of PPAR-α in the liver of KKA^y mice. These effects could be additives to the apetite controling effect of *V. vitis*.

In the course of bioguided-fractionation, caffeic acid methyl ester (CAME), a by-product of fractionation procedure, has been shown to potently stimulate glucose uptake in cultured skeletal muscle cells and therefore to have anti-diabetic potential. To identify other active caffeic acid (CA) derivatives and to elucidate their structure–activity and structure-toxicity relationships, twenty CA derivatives were tested. In addition to CAME, four compounds were found to stimulate glucose uptake and activate AMPK. Uncoupling of mitochondrial oxidative phosphorylation by these compounds resulted in metabolic stress which could explain the activation of AMPK. The activity required an intact caffeic acid moiety devoid of strongly ionized groups and was well correlated with lipophilicity and toxicity.

The results of the present thesis support a therapeutic potential for *V.vitis*, and its active compounds, as well as the CA family of compounds for the prevention and treatment of diabetes.

Keywords: *Vaccinium vitis*, type 2 diabetes, AMPK, ACC, PPAR-α, OPD, KKA^y, GLUT4, natural health products, traditional medicine, Canadian boreal forest, Aboriginal populations of North America.

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List of abbreviations

µg: Microgram

µL: Microliter

ACC: Acetyl-CoA carboxylase

AICAR: aminoimidazole carboxamide ribonucleotide

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ATP: Adenosine triphosphate

CA: Caffeic acid

CAM: Complementary and alternative medicine

CAME: Caffeic acid methyl ester

CAP: Cbl-associated protein

CAPE: Caffeic acid phenethyl ester

CEI: Cree of Eeyou Istchee

cm: Centimeter

DMSO: Dimethyl sulfoxide

DPP-4: Dipeptidylpeptidase 4

FFAs: Free fatty acids

g: Gram

G-6-P: Glucose 6-phosphate

G-6-Pase: Glucose 6-phosphatase

GLUT: Glucose transporters

GS: Glycogen synthase

GSK-3: Glycogen synthase kinase 3

h: Hour

HPLC: high performance liquid chromatography

IGT: Impaired glucose tolerance

IL: Interlukin

IR: Insulin receptor

IRS: Insulin receptor substrate family

Kg: Kilogram

LDL: Low density lipoprotein

MAPK: mitogen-activated protein kinase

MC4-R: melancortin receptor 4

min: Minute

mL: Milliliter

NMR: Nuclear magnetic resonance

NPY: Neuropeptide Y

OGTT: Oral glucose tolerance test

OPD: *O*-phenylenediamine dihydrochloride

PDK1: 3-phosphoinositide-dependent protein kinase-1

PEPCK: Phosphoenolpyruvate carboxykinase

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator-1

PKC: Protein kinase C

PPAR: Peroxisome-proliferator–activated receptor

PTB: Phosphotyrosine-binding

PTPases: Protein tyrosine phosphatases

RASOC: the rate of ADP-stimulated O_2 consumption

RBOC: the rate of basal oxygen consumption

SGLT: Sodium glucose co-transporters

SOS: Son of Sevenless

SREBPs: Sterol regulatory element-binding proteins

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

TNF-α: Tumor necrosis factor

TZDs: Thiazolidinediones

UCPs: Uncoupling proteins

UV: Ultraviolet

VLDL: Very low density lipoprotein

To my family

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1. Introduction

The last few decades of the 20th century have witnessed the rise of worldwide epidemic obesity along with type 2 diabetes mellitus in adults, as well as in children and adolescents. Type 2 diabetes is a metabolic disorder characterized by hyperglycemia. Its prevalence varies among different ethnic groups. In Canada, the overall prevalence of diabetes among Canadian adults was approximately 5.5% in 2004-2005 according to National Diabetes Surveillance System (NDSS) data. The populations most affected are the aboriginal populations with a higher prevalence in women compared to men. The recent socio-cultural changes experienced by these populations including adoption of a sedentary lifestyle, the consumption of non-traditional foods, along with the genetic predisposition to the disease are the major causes of the epidemic (Boston *et al.* 1997); (Young et al., 2000); (Hegele, 2001). Aboriginals also suffer from diabetes complications, including end-stage renal failure, retinopathy and peripheral neuropathy, at a disproportionately high rate. It was reported that death from diabetes complications is 5-fold higher among aboriginal women as compared with Canadian women (Young et al., 2000).

The Cree represent the largest aboriginal group in Canada, with more than 72,000 registered individuals (Statistics Canada, 2002). Eeyou Istchee, which literally means “land of the people”, is the homeland of the Cree Nation of Eastern James Bay. The Cree of Eeyou Istchee (CEI) have a population of approximately 14,000 people who live in 9 communities spread across the northern part of the province of Quebec (Secrétariat aux affaires autochtones, 2004). Over the past decade, diabetes has reached unprecedented proportions among the CEI with a prevalence of 17.7% among adults aged over 20 years (Légaré, 2004). A high prevalence of gestational diabetes has been also observed in CEI, which ranks second among aboriginal groups worldwide (Rodrigues et al., 1999).

To address this serious aboriginal health issue, diabetes prevention and treatment projects need to be adapted to the cultural and social environment of these populations. Aboriginal peoples have a long tradition of using plants in their environment for healing purposes; through millennia of trial and errors they had developed a comprehensive

traditional pharmacopeia that was handed down from generation to generation largely as verbal teaching and as part of their cultural tradition (Young et al., 2000).

After the European colonization, some of their traditional knowledge was lost; fortunately much was documented by anthropologists and was recognized in the official Pharmacopeia of the United States and Canada. The first Pharmacopoeia of the United States, published in 1820, included 170 indigenous plant species. Similarly, the Canadian Pharmaceutical Journal contained more than twenty species prescribed by First Nations as medication (Erichsen-Brown, 1979; Vogel, 1990).

Despite the wealth of the Cree Nations' traditional knowledge, no work has been done to examine the potential of their medicinal plants to treat the relatively recent diabetes epidemic. The approach that our research team has adopted was to develop culturally relevant diabetes treatment options in these communities. Taking into consideration the complexity of diabetes and its relatively recent evolution among the Cree population, the ethnobotanical approach adopted by our project was based on the the symptoms and complications of the disease. Hence a questionnaire that included 15 symptoms of type 2 diabetes, rather than diabetes per se was prepared. In collaboration with the CEI, we have conducted ethnobotanical surveys in four CEI communities: Mistissini, Whapmagoostui, Nemaska and Waskaganish and have identified seventeen medicinal plant species that are traditionally used to treat symptoms related to diabetes (Leduc et al., 2006), (Fraser et al., 2007), (Harbilas et al., 2009). Bioactivity screening projects for antidiabetic properties of these plant products have showed that over half of them possess significant antidiabetic activities (Spoor et al., 2006), (Harbilas et al., 2009). The present study focuses on the antidiabetic properties of the berries of *Vaccinium vitis-idaea*, a medicinal plant product used in the communities of Whapmagoostui and Mistissini to treat frequent urination and a number of other symptoms of diabetes (Leduc et al., 2006); (Fraser et al., 2007).

1.1 Energy homeostasis

1.1.1 Glucose homeostasis

Glucose is the primary metabolic fuel for all body tissues and the obligatory energy substrate for the brain. Despite the relatively small weight of the brain (2% of body weight), it uses 25% of the total body glucose (Dong et al., 2003). Therefore, a constant and adequate supply of glucose is necessary to maintain normal brain function (Tirone and Brunicardi, 2001). During fed and fasting states, healthy individuals are able to maintain plasma glucose in narrow ranges (fasting blood glucose of 3.3–5.6 mmol/L and post-prandial glucose of 4.40–6.94 mmol/L). To keep this tight control of blood glucose concentration, a balance should be achieved between glucose absorption by the intestine, glucose production by the liver and glucose disposal in peripheral tissues (Beardsall et al., 2008). Insulin is the master regulator of blood glucose level in the fed state and does so by controlling glucose uptake by muscle and fat cells and by suppressing hepatic glucose production. On the other hand, glucagon and other counter-regulatory hormones (catecholamines, cortisol and growth hormone) maintain blood glucose levels during fasting (Beardsall et al., 2008).

1.1.1.1 The insulin receptor: transduction through tyrosine kinase signaling

The insulin receptor (IR) is a heterotetrameric plasma membrane protein receptor that consists of two extracellular α- and two intracellular β-subunits linked by disulfide bonds. It belongs to a subfamily of receptor tyrosine kinases that also includes the insulin-like growth factor and an orphan receptor, known as the IR-related receptor (Ward, 1999). Before ligand binding, IR is inactive, although it is oligomerized, since the α subunit exhibits allosteric inhibition of the β subunit catalytic activity. Binding of the ligand (insulin) induces a conformational change which stimulates the catalytic activity and induces transphosphorylation of the receptor on specific tyrosine residues. Once phosphorylated, these tyrosine residues, along with the sequence of adjacent amino acids, create binding sites for docking proteins, which contain domains that bind phosphotyrosine such as Src homology 2 domain (SH2) and phosphotyrosine binding (PTB). Scafolds

(insulin receptor substrate (IRS), Gab, and Shc), adaptors (Grb2), kinases (phosphatidylinositol 3-kinase (PI3-K), Src), phosphatases (Shp2) and ubiquitinating proteins (c-Cbl) are recruited to the phosphorylated active IR through these domains to be phosphorylated by the catalytic activity of IR on tyrosine residues (Baron et al., 1992), (Saltiel and Kahn, 2001). This links IR to several major signaling pathways, one involves phosphatidylinositol 3-kinase (PI3-K) and the other involves Ras/mitogen-activated protein kinase (MAPK) cascade (Figure 1).

1.1.1.1.1 PI3-Kinase pathway and downstream targets

This pathway is responsible for the metabolic action of insulin. Recruitment of PI3-K to IRS through its regulatory subunit, p85, results in the phosphorylation of phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) by PI3-K catalytic subunit, p110, which generates phosphatidylinositol (PI(3,4,5)P3). PI(3,4,5)P3 serves as docking site for pleckstrin homology domain (PH) containing serine/threonine kinase Akt/PKB. Once correctly positioned at the membrane, Akt gets phosphorylated by its activating kinases, phosphoinositide-dependent protein kinase-1 (PDK1), at threonine 308 (Jacob et al., 2008). PDK1 also phosphorylates and activates the atypical forms of protein kinase C (PKC), including PKCξ and PKCλ. Both Akt and atypical PKC have been shown to mediate insulin dependent glucose transport (Czech and Corvera, 1999). This is discussed in detail further below.

1.1.1.1.2 The Ras–MAPK cascade

The Ras-MAPK pathway is mainly involved in mediating cell growth, survival and cellular differentiation. Phosphorylation of IRS-1 by IR induces the translocation of cytosolic adaptor protein, growth factor-bound protein 2 (Grb-2) through its SH2 domain. Grb2 also contains SH3 domain which allows the constitutive association with the proline rich region of the guanyl nucleotide exchange factor, son of sevenless (SOS). Alternatively, Grb2/SOS complex is recruited to IR through the assistance of another adaptor, Shc. The recruitment of Grb2 from the cytoplasm to the plasma membrane binds the small GTPase Ras. Through guanine exchange, SOS activates Ras, thus allowing its

interaction with downstream effectors and their activation. Ras initiates a kinase cascade via the stepwise activation of Raf, the MAP kinase-kinase MEK and the MAP kinases ERK1 and ERK2. Once activated, ERKs promote gene expression and protein synthesis by phosphorylating targets such as p90 ribosomal protein S6 kinase (p90RSK) and the transcription factor ELK1 (Avruch, 1998).

1.1.1.1.3 Cbl/CAP/TC10 pathway in lipid rafts

The insulin receptor also phosphorylates other substrates such as the Cbl-binding protein APS. Phosphorylation of APS is necessary for its binding with Cbl and the subsequent phosphorylation of Cbl by IR. Cbl interacts also with Cbl-associated protein (CAP), a protein that belongs to the Sorbin homology (SoHo) family of adaptor proteins. The phosphorylated APS-Cbl–CAP complex then translocates to lipid raft domains in the plasma membrane through the interaction of the SoHo domain of CAP with the lipid raft protein, flotillin. Once translocated into the lipid raft, the phosphorylated Cbl recruits the SH2/SH3 adaptor protein CrkII through its SH2 domain along with the guanyl nucleotide-exchange protein C3G. C3G, in turn, catalyze the exchange of GTP for GDP on the lipid-raft-associated protein TC10, a Rho family GTPase, resulting in its activation. Along with the PI3-K, TC10 stimulates the trafficking of Glut4 vesicles, their docking and their fusion with the plasma membrane (Saltiel and Kahn, 2001), (Kimura et al., 2002). This pathway was first described in 3T3-L1 adipocytes, but was later reported in cardiac muscle and adipose tissue *in vivo* (Gupte and Mora, 2006). Another group suggested that the Cbl/CAP/TC10 signaling cascade was present in skeletal muscle, activated by insulin, and impaired by high-fat feeding (Bernard et al., 2006).

1.1.1.1.4 Regulation and termination of IR signal

Given the important biological functions of IR, its signal must be tightly regulated. This occurs by terminating IR signaling through its internalization and dephosphorylation by protein tyrosine phosphatases (PTPases) such as PTP1B. IR is then ubiquitinated and degraded by the proteasome (Zinker et al., 2002), (Saltiel and Kahn, 2001).

Alternative regulation of IR could be the result of cross talk signaling from other receptors such as the epidermal growth factor receptor (EGFR), tumor necrosis factor alpha (TNF-α) and integrin receptors. IR has been shown to be phosphorylated at Ser/ Thr residues, which results in the attenuation of insulin signaling (Coba et al., 2004). Finally, IRS-1 is also the target of Ser/ Thr phosphorylation by PKC-β, which results in the inhibition of the catalytic activity of IR (Aguirre et al., 2002) (Liberman et al., 2008) (Ishizuka et al., 2004). IRS can also be negatively regulated by kinases mediators of insulin signalling upon prolonged insulin stimulation such as PKCξ,(Liu et al., 2001), mTOR/S6K1 (Tremblay et al., 2007) and certain MAPK (Engelman et al., 2000). Novel members of PKC family (nPKCs) such as PKC-ε, -η and –θ can directly phosphorylate IRS-1 on serine/therionine residues. nPKCs are involved in free fatty acids (FFAs)-induced insulin resistance. The metabolism of FFA increases the content of intramyocellular diacylglycerol (DAG), a potent allosteric activator of conventional and novel PKCs (Dey et al., 2006).

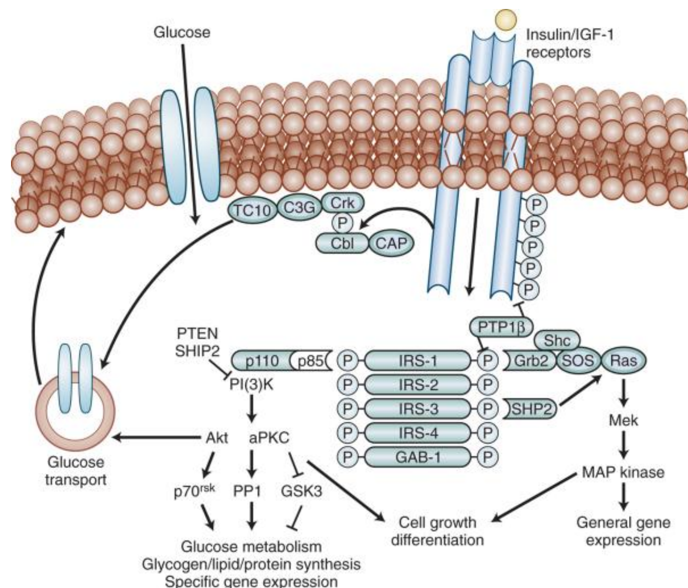

Figure 1 Insulin signaling pathways (Saltiel and Kahn, 2001)

1.1.1.2 Glucose transporters and insulin action

In the fed state, insulin promotes glucose disposal mainly through glucose uptake by peripheral tissues and its subsequent utilization/storage. Because glucose is a polar

molecule, it does not diffuse through the lipid bilayer of cell membranes and therefore glucose transporters are required (Olson and Pessin, 1996). Two different types of glucose transporters have been identified: the energy dependent sodium glucose co-transporters (SGLT) and the facilitative glucose transporters (GLUT). SGLT are expressed mainly in the intestine and kidney, where they actively transport glucose against its concentration gradient by using the energy derived from the co-transport of sodium down its electrochemical gradient (Shepherd and Kahn, 1999).

GLUT transporters are a family composed of 14 structurally related proteins that mediate facilitative transport of glucose along its concentration gradient. These proteins have 12 membrane spanning domains with both C- and N- terminal tails of the protein oriented on the cytoplasmic side (Augustin, 2010; Olson and Pessin, 1996; Thorens and Mueckler, 2009; Wood and Trayhurn, 2003). They are encoded by distinct genes and have distinct substrates and tissue distribution (Table 1). GLUT family can be grouped into three classes bases on their structural similarities, class I that includes GLUT1-4 and GLUT14, class II comprises GLUT5, 7, 9, 11, and class III which has GLUT6, 8, 10, 12 and the proton driven myoinositol transporter HMIT (or GLUT13). GLUT4 is the main insulin-responsive glucose transporter and is located primarily in skeletal muscle cells, cardiac muscle cells and adipocytes (James et al., 1989). In these tissues, GLUT4 is responsible for most of the glucose uptake, even though they also express the GLUT1 isoform (Giorgino et al., 2000). In the basal state, less than 5% of GLUT4 resides in the cell surface, the rest are present in the membrane of the vesicles. Upon insulin stimulation, GLUT4 is translocated to the cell surface and glucose uptake is increased (Cushman et al., 1998), (Suzuki and Kono, 1980), (Marette et al., 1992).

Table 1 Distribution of mammalian facilitative glucose transporters (Augustin, 2010; Thorens and Mueckler, 2009)

Name	Tissue distribution	Insulin sensitivity	Function
GLUT1	Ubiquitous, erythrocytes and brain	No	Basal glucose transport, transport across blood-brain barrier
GLUT2	Liver, pancreatic β-cells, intestine, kidney	No	Intestinal absorption, renal re-absorption, pancreatic and hepatic control of glucose homeostasis
GLUT3	Widely distributed in human tissues, restricted to brain in other species. Immune cells.	No	Glucose transport into neurons in brain, basal transport in many human cells.
GLUT4	Skeletal muscle, cardiac muscle, adipose tissue	Yes	Insulin-dependent glucose transport.
GLUT5	Intestine, testes, kidney.	No	Fructose transport.
GLUT6	Leucocytes, spleen, brain.	No	n. d.
GLUT7	Apical membrane of small and large intestine	No	Exhibits a low level of transport activity for fructose and glucose.
GLUT8	Brain, heart	Yes	Role in neuronal proliferation and heart atrial activity.
GLUT9	Liver, kidney, intestine	No	Regulator of uric acid liver.
GLUT10	Liver, pancreas	No	n. d.
GLUT11	Different tissue types	No	Glucose, fructose transport, main substrate has not been identified.
GLUT12	Heart, prostate, musle, small intestine, WAT	Yes	Glucose homeostasis.
GLUT13	Brain	No	Myoinositol transporter.
GLUT14	Testis	No	Most likely a glucose transporter.

Once inside muscle, glucose is phosphorylated by hexokinase or glucokinase, yielding glucose 6-phosphate (G-6-P). G-6-P can be then used either for the synthesis of glycogen via the activity of glycogen synthase (GS) or metabolized in the glycolytic pathway via enzymes such as pyruvate kinase (Roach, 2002).

1.1.1.3 Alternative pathway to glucose uptake: AMP-activated protein kinase pathway

AMP-activated protein kinase (AMPK) is a ubiquitous heterotrimeric enzyme composed of α, β and γ subunits. The α subunit contains a serine/threonine protein kinase catalytic domain. It also holds Thr/172 which is the principal site of its phosphorylation by upstream kinases and thus its activation. The β subunit has glycogen-binding domains and appears to stabilize the interaction between α and γ subunits. The γ subunit binds AMP or ATP in a mutually exclusive manner (Wong and Lodish, 2006).

AMPK is a metabolic stress-sensing protein kinase that is activated in response to depletion of ATP content and an increase in the cellular AMP/ATP ratio under stress conditions such as hypoxia, physical exercise and inhibition of mitochondrial respiration (Chen et al., 1999). AMP is thought to allosterically activate the enzyme and prevent its dephosphorylation. On the other hand, the tumor suppressor LKB1 is the major kinase responsible for phosphorylation of AMPK on Thr^{172} in the α-subunit. However, LKB1 is not directly activated by AMPK, but the later induces conformational changes in AMPK rendering it more susceptible to phosphoylation by LKB1. Ca^{2+}/calmodulin-dependent almodulin protein kinase kinase (CAMKK) can also phosphorylate AMPK on Thr^{172} and activate it (Shen et al., 2007). When AMPK is activated, energy-consuming anabolic pathways are shuts down (such as fatty acid, protein and cholesterol synthesis) and ATP-producing catabolic pathways are activated (such as fatty acid oxidation) (Viollet et al., 2003).

Within skeletal muscle, the activation of AMPK increases glucose uptake through the stimulation of GLUT4 translocation to the plasma membrane by a mechanism distinct

from PI3-K stimulated by insulin. P38 MAPK appears to be activated by several stimulators of AMPK, including AICAR and mitochondrial uncouplers, and is thought to mediate the effect of AMPK on activation of GLUT4 exposed at the cell surface (Lemieux et al., 2003; Pelletier et al., 2005; Ribe et al., 2005). In non-insulin sensitive tissues, AMPK stimulates glucose transport by activating GLUT1 at the plasma membrane. In addition, AMPK upregulates the expression of GLUT4 possibly through the direct phosphorylation of the transcriptional co-activator PPARγ coactivator-1 (PGC 1α) or through the derepression of the transcription factor myocyte enhancer factor-2 (MEF2). Moreover, activation of PGC-1α by AMPK also increases the mitochondrial biogenesis resulting in greater mitochondrial oxidative capacity. Thus, AMPK activation might protect against mitochondrial dysfunction thought to predispose to metabolic diseases such as obesity and type 2 diabetes (Lowell and Shulman, 2005).

On the other hand, AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC). There are two isoforms of ACC in mammalian tissues: ACC1 and ACC2. ACC1 is a cytosolic protein involved in fatty acid synthesis, while ACC2 exists mainly in the mitochondria and regulates fatty acid oxidation. Collectively, inhibition of ACC leads to reduction of malonyl-CoA concentrations, an allosteric inhibitor of carnitine palmitoyl transferase (CPT-1). The later enzyme regulates the transport of long chain fatty acids to mitochondria for β-oxidation. Thus, the derepression of CPT-1 by ACC phosphorylation decreases intramyocyte accumulation of lipids and increases insulin sensitivity of muscle (Winder and Hardie, 1999), (Zhou et al., 2009) (Fogarty and Hardie, 2010).

In a similar manner, activation of AMPK in the liver stimulates fatty acid oxidation and inhibits expression of genes encoding lipogenic enzymes (fatty acid synthase and ACC) (Viollet et al., 2003). The action of AMPK on the lipogenic genes is mediated by reduction of transcription activators namely carbohydrate responsive element-binding protein (ChREB) and sterol regulatory element-binding protein 1c (SREPB-1c). In addition, AMPK inhibits the synthesis of cholesterol via the suppression of 3-hydroxy-methyl-glutaryl-CoA reductase (HMGR).

AMPK also decreases hepatic glucose production mainly by inhibiting the expression of gluconeogenic genes such as phosphoenolpyruvate carboxylase (PEPCK) and Glucose 6-phosphate (G-6-Pase). The action of AMPK on the expression of these genes involves regulation of transcription factors including cAMP-response element-binding protein (CREB), hepatocyte nuclear factor-4α (HNF4-α), Forkhead box O1(FOXO1), and the orphan nuclear receptor small heterodimer Partner (SHIP). The metabolic actions of AMPK are summarized in Figure 2.

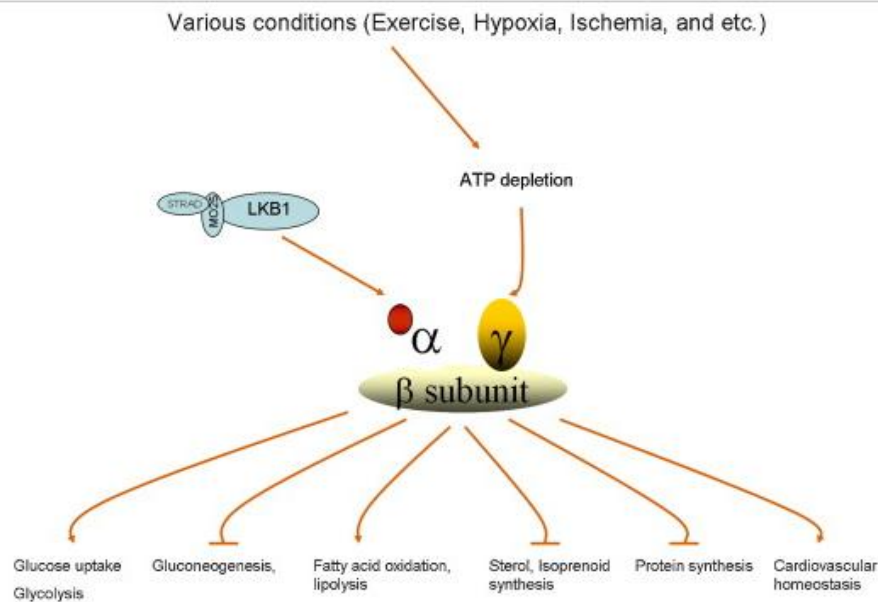

Figure 2 major effects of AMPK on glucose and fatty acid metabolism in liver and skeletal muscle (Hwang et al., 2009).

It has been reported that the insulin-sensitizing drugs thiazolidinediones and biguanides, though chemically unrelated, exert part of their effects through regulation of the activity of AMPK (Fryer et al., 2002). Biguanides such as metformin and phenformin are reported to be transported by the organic cation transporter-1 (OCT1) which is highly expressed in liver. The detailed mechanisms of these agents will be discussed under treatment section. On the other hand, the synthetic nucleotide analogue nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAR) has been widely used to study the effect

of AMPK in animals. Inside the cell, AICAR is metabolized to the monophosphorylated derivative ZMP, which mimics the effect of AMP on AMPK activation. Interestingly, direct activators of AMPK such as A-769662 and PT1 have been recently developed. The action of these agents does not seem to be mediated by AMP. Activation of AMPK by A-769662 appears involve the β-subunit (Fogarty and Hardie, 2010)

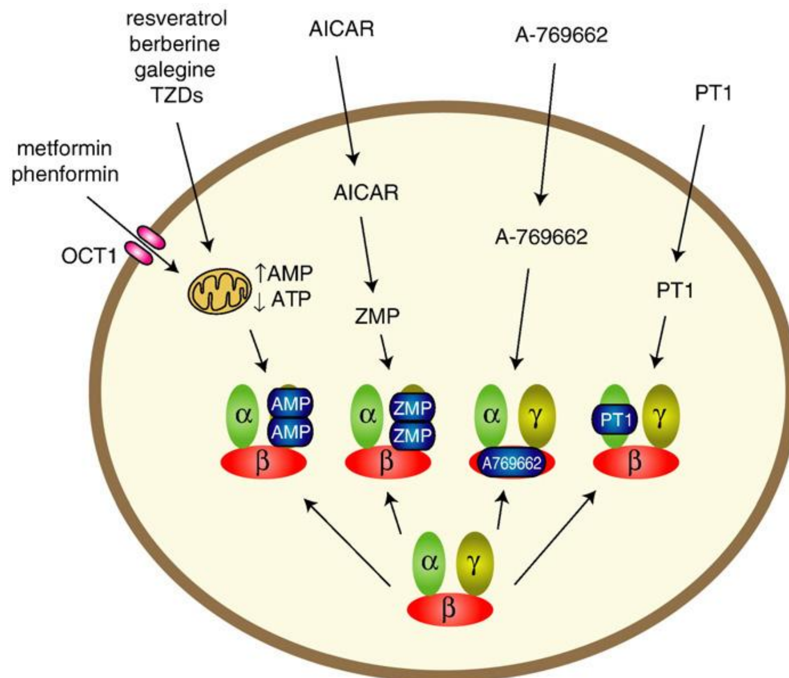

Figure 3 : Activators of AMPK (Fogarty and Hardie, 2010)

1.1.1.4 Regulation of hepatic glucose production

The liver is responsible for maintaining glucose homeostasis by achieving a balance between the uptake and conversion of glucose to glycogen (glycogenesis) on the one hand, and the release of glucose by breaking down glycogen (glycogenolysis) or by de novo synthesis of glucose (gluconeogenesis), on the other hand (Nordlie et al., 1999). In gluconeogenesis, glucose is synthesized from non-carbohydrate precursors such as lactate, glycerol, and alanine. The enzymes controlling these processes are mainly regulated at the

level of gene transcription by several hormones, principally glucagon and insulin (Shao et al., 2005).

In the fasting state, insulin levels drop while glucagon levels rise. Indeed, glucagon enhances glycogenolysis and gluconeogenesis in the liver, while inhibiting glycogenesis to increase glucose production and maintain blood glucose levels stable (Aronoff et al., 2004). Similarly, growth hormone and cortisol regulate blood glucose levels during fasting by stimulating lipolysis, which increases levels of circulating FFAs and glycerol. This glycerol, once transported into the liver is phosphorylated and converted into glucose (Beardsall et al., 2008).

In contrast, during fed state and in response to rising blood glucose levels, insulin stimulates glycolysis by increasing gene transcription of glucokinase and pyruvate kinase. In parallel, insulin decreases gene transcription of gluconeogenic enzymes, fructose 1,6-bisphosphatase and the rate limiting enzymes, PEPCK and G-6-Pase. In addition insulin exerts an indirect control on gluconeogenesis by decreasing substrate availability (Beardsall et al., 2008), (Barthel and Schmoll, 2003).

Finally, in the liver, as in the muscle, insulin induces glycogenesis. Indeed, insulin-stimulated activation of Akt results in the dephosphorylation and the activation of glycogen synthase (GS) through the inhibition of glycogen synthase kinase 3 (GSK-3) following its phosphorylation (Bouskila et al., 2008). Parallel to that, insulin inhibits liver glycogen phosphorylase activity, the enzyme catalzing glycogenolysis (Petersen et al., 2001).

1.1.2 Regulation of lipogenesis and lipolysis

As is the case with glucose metabolism, insulin is also a key hormone regulating lipid metabolism. It does so by controlling both lipogenesis and lipolysis in adipose tissue. It promotes the activity of lipoprotein lipase (LPL), the enzyme that hydrolyzes lipids in lipoproteins such as chylomicrons and very low density lipoprotein (VLDL), and its secretion by the liver (Pradines-Figueres et al., 1988). Simultaneously, insulin inhibits the activity of hormone-sensitive lipase (HSL), the key enzyme of lipolysis in the adipocytes, thereby limiting the release of fatty acids and glycerol and allowing the storage of

triglycerides (Kraemer and Shen, 2002). Moreover, insulin enhances the activity of enzymes involved in lipid synthesis including pyruvate dehydrogenase, fatty acid synthase and ACC (Moustaid et al., 1996). Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate enzymes required for cholesterol and fatty acid synthesis. Recent studies showed that expression of SREBP-1 was enhanced by insulin in liver, fat, and skeletal muscle, the three major insulin-sensitive tissues (Nadeau et al., 2004).

1.2 Diabetes mellitus

1.2.1 Definition and diagnosis

Diabetes mellitus is a worldwide epidemic currently affecting 246 million people and that number is expected to rise to 380 million by 2025 (Levitt, 2008). It is characterized by increased circulating glucose concentration associated with abnormalities in the metabolism of carbohydrate, fat and protein (WHO 1999). Diabetes stems from inadequate insulin secretion, from the failure of the body to respond to insulin normally or from both (Cavaghan et al., 2000). Patients presenting symptoms of diabetes such as polyuria, thirst, unexplained weight loss, fatigue and in severe cases drowsiness or coma can be diagnosed for diabetes by monitoring their fasting blood glucose levels. In healthy subjects, fasting blood glucose levels should be less than 100 mg/ dL (5.5 mmol/ L). Subjects with fasting glucose levels above those values are very likely to have either impaired glucose tolerance (IGT) or diabetes and should undergo an oral glucose tolerance test (OGTT). OGTT should be preceded by 10 to 16 hours overnight fast and blood samples are drawn at fasting and during the 2 hours following a 75 g oral glucose load. A fasting glycemia of 126 mg/dl (7 mmol/L) or above as well as a glycemia of 200 mg/ dl (11.1 mmol/L) after 2 hours following an OGTT confirms diagnosis of diabetes (WHO 1999).

1.2.2 Diabetes Classification

The classification of diabetes has changed considerably over the past three decades. In 1980, the WHO proposed two major classes of diabetes mellitus and named them type 1

diabetes or insulin dependent diabetes mellitus (IDDM) and type 2 diabetes or non insulin-dependent diabetes mellitus (NIDDM). Both the 1980 and 1985 WHO reports recognized other classes of diabetes including Gestational Diabetes Mellitus (GDM) and Other Types Diabetes. As knowledge regarding the pathophysiology of diabetes evolved, a newer classification based on the etiology of the different forms of diabetes has been adopted. The etiological classification of diabetes mellitus currently recommended by WHO and the American Diabetes Association (ADA) recognize two broad etiopathogenetic categories, now called type 1 and type 2 diabetes. The terms IDDM and NIDD which classified patients based on treatment are no longer used (Gavin et al. 1997, WHO). While continuing to recognize gestational diabetes independently, other specific types of diabetes, secondary to or associated with specific diseases or with a distinct etiology, have been categorized under “Other specific types” (WHO, 1999).

1.2.2.1 Type 1 diabetes mellitus (T1DM)

T1DM is responsible for 5-10% of all cases of diabetes. This form of disease encompasses the cases that are primarily due to β-cell destruction with autoimmune or idiopathic origin and are ketoacidosis-prone. It does not include forms of β-cell destruction with specific causes (e.g. cystic fibrosis) (WHO 1999). Patients who have T1DM are metabolically normal before the disease is clinically diagnosed (Kahn and Saltiel, 2005).

T1DM is usually characterized by the presence of auto-antibodies against islet cells or against insulin. Individuals who have one or more of these antibodies are classified under type 1A diabetes. On the other hand, some forms of type 1 diabetes have no known etiology or clinical evidence of autoimmune antibodies and are classified under the term type 1B or idiopathic diabetes (WHO, 1999, (Tanaka et al., 2000), (Kahn and Saltiel, 2005). A major characteristic of T1DM is the dependence on exogenous administration of insulin due to an absolute deficiency of insulin (WHO 1999).

1.2.2.2 Type 2 Diabetes mellitus (T2DM)

T2DM accounts for approximately 90-95% of cases diagnosed with diabetes worldwide. It results from insufficient insulin secretion, combined with insulin resistance

(WHO 2003). These individuals might not require insulin treatment, although many end up needing it to maintain blood glucose control. Obesity, family history and physical inactivity increase the risk of developing T2DM (Oguma et al., 2005). Until recently, T2DM was considered as adult-onset disease. However, evidence is accumulating that, as a consequence of the current epidemic of obesity, diabetes among children and adolescents is becoming increasingly apparent (Copeland et al., 2005).

1.2.2.3 Gestational diabetes mellitus (GDM)

This term refers to hyperglycemia first appearing or first being diagnosed during pregnancy whether or not insulin treatment is adopted or diabetes persists after pregnancy. In Canada, GDM affects 3.7% of non-Aboriginal women and 8–18% of Aboriginal pregnant women (Rodrigues et al., 1999). GDM is considered a risk factor for developing T2DM (Kim et al. 2005).

1.2.2.4 Other Specific Types of diabetes

Sometimes referred to as secondary diabetes, these types are less common causes of diabetes mellitus. This category comprises a variety of conditions, in which the molecular defects are well defined or the underlying disease process can be identified (WHO 1999). An example is β-cell dysfunction associated with specific monogenetic defects known as MODY, or maturity onset diabetes of the young, since the onset of hyperglycemia occurs at a young age (Hattersley, 1998).

1.2.3 Pathogenesis of type 2 diabetes

T2DM is a polygenic disease where genetic defects underlie insulin resistance and insulin insufficiency, the two major players in the pathogenesis of T2DM. For many years, β-cell dysfunction was thought to be secondary to increased secretory demands induced by insulin resistance. However, it is now well established that the two processes evolve in parallel, more or less independently of one another (Saltiel and Kahn, 2001). Indeed, environmental factors and life style including consumption of high-calorie foods, physical inactivity, and obesity contribute to the development of the disease (Hamman, 1992).

Toxicities from hyperglycemia (glucotoxicity) and FFAs (lipotoxicity) affect β-cell function and further aggravate insulin resistance. The net outcome is the impairment of basal and stimulated insulin secretion, the dysregulation of glucose production by the liver and of glucose uptake by muscle (Unger, 1995), (Weyer et al., 1999).

1.2.3.1 Insulin resistance

Insulin resistance is a condition in which normal levels of insulin do not generate a normal biological response. Because one of insulin's major physiological actions is to maintain glucose homeostasis, a rise in the fasting plasma glucose level can eventually occur (Krentz, 1996). If the pancreatic islet is normal, it will compensate for the insensitivity to insulin by increasing β-cell insulin secretion, with ensuing hyperinsulinemia. During progression towards diabetes, pancreatic β-cells can no longer produce enough insulin and glucose levels rise relatively rapidly (Bonner-Weir, 2000).

The metabolic syndrome, or syndrome X, is a cluster of three or more abnormalities that includes obesity (notably, abdominal adiposity measured by waist circumference; see next section), dyslipidemia (hypertriglyceridemia and hypercholesterolemia), high fasting plasma glucose and hypertension (Reaven, 2002). Insulin resistance is the center of these abnormalities and is more common in obese subjects. Therefore, it is considered a risk factor for the development of both type 2 diabetes and cardiovascular diseases (Silfen et al., 2001). As a matter of fact, 90% of individuals with type 2 diabetes are either overweight or obese (Torgerson et al., 2004). As the association between obesity and insulin resistance has been reported in both normoglycemic and diabetic individuals, it is now believed that a strong connection exists between insulin resistance in T2DM and increased adiposity (Steinberger and Daniels, 2003).

1.2.3.1.1 Body-Fat distribution and insulin resistance

The distribution of fat rather than the degree of obesity appears to be an important indicator for the metabolic complications of obesity. Fat accumulated around abdominal organs, referred to as central obesity, is more metabolically active than subcutaneous fat. As a result, there is a greater influx of FFAs and increased accumulation of triglycerides in

non-adipose tissues (ectopic fat). This ectopic fat aggravates insulin resistance since intramyocellular triglycerides (IMTG) inhibit insulin-stimulated glucose uptake in the muscle (Shulman 2002). In the liver, increased delivery of FFAs leads to increased hepatic glucose output and increased hepatic insulin resistance (Gastaldelli et al., 2007). FFAs accumulation in the pancreas leads to accumulation of toxic fatty acid metabolites inducing apoptosis and β-cell dysfunction (Lencioni et al. 2008). Finally, intra-abdominal fat is also more active in producing adipocyte-secreted hormones (adipokines) and inflammatory cytokines than is subcutaneous fat (Xu, 2003).

1.2.3.1.2 Secretory function of adipose tissue

White adipose tissue (WAT) was once thought to be an inactive storage tissue. Over the past few years, however, it was found to be an active secretory organ producing many hormones. These are referred to as adipokines because they are secreted by the adipocytes; leptin, adiponectin and resistin are prominent examples. WAT also secretes a large variety of other cytokines such as interleukine-6 (IL-6) and its receptor antagonist (IL-1Ra) as well as TNF-α (Fain et al., 2004), (Juge-Aubry et al., 2005). Under normal physiological conditions adipokines play an important role in regulating inflammation, glucose and lipid metabolism, as well as contributing to the maintenance of energy homeostasis. In obesity, and particularly in central obesity, however, they contributed to related metabolic and vascular complications (Kusminski et al., 2005).

1.2.3.1.2.1 Leptin

Leptin is the product of ob/ob gene and is secreted mainly by WAT. It acts on receptors in the brain and other tissues to diminish food intake and increase energy expenditure thus decreasing body weight (Meli et al., 2004). Leptin action is mediated through AMPK pathway, which can be activated either directly by leptin or indirectly through the hypothalamic-sympathetic nervous system axis (Minokoshi et al., 2002). Leptin stimulates fatty acids oxidation and increases glucose uptake, and prevents ectopic lipids accumulation in non-adipose tissues, thereby enhancing insulin sensitivity. Although

circulating leptin levels increase in obesity, which should result in better insulin response, obese individuals were found to be resistant to leptin action (Zimmet et al., 1996).

1.2.3.1.2.2 Adiponectin

Adiponectin is a hormone with potent insulin sensitizing properties, whose expression was though to be restricted to adipose tissue (Ziemke and Mantzoros). Interestingly, human and murine cardiomyocyte were found to express adiponectin and its receptors (Ding et al., 2007; Guo et al., 2007; Pineiro et al., 2005). Recently, the human vascular smooth muscle cells (VSMC) of coronary artery were also reported to express and secrete adiponectin (Ding et al., 2010). In contrast to the other adipokines (e.g. leptin and resistin), circulating adiponectin levels are reduced in obese, insulin-resistant humans. Indeed, adiponectin expression correlates negatively with visceral adipose tissue in either lean or obese subjects (Lihn et al., 2004). In addition, experimental studies in animals and humans have shown that insulin-sensitizer such as thiazolidinediones substantially increase adiponectin concentrations (Lindsay et al., 2002). A similar action has recently been found with a fermented blueberry juice prepared in our laboratory (Vuong et al., 2009).

Similar to the action of leptin, the insulin-sensitizing properties of adiponectin can be attributed, at least in part, to activation of AMPK in skeletal muscles and adipocytes, thereby increasing fatty acids oxidation and increasing glucose uptake. Furthermore, adiponectin activates AMPK in the liver, resulting in reduced rate of hepatic glucose production (Kadowaki et al., 2008). Therefore, the decrease in adiponectin levels in insulin resistance further aggravates hyperglycemia.

1.2.3.1.2.3 Resistin

This hormone discovered in 2001 appears to be a pro-inflammatory cytokine that has a potential role in inflammation and immunity (Tilg and Moschen, 2006). Murine resistin consists of 114 amino acids, while human resistin is composed of 108 amino acids. However, although the expression of resistin in mice was originally detected in the adipocytes, it has also been detected in the pituitary glands, the hypothalamus and in the blood circulation. Conversely, in humans, resistin is highly expressed in the bone marrow

and to a much lesser extent in the adipose tissue where its expression is confined to non-fat-stroma-vascular fraction. It is also found in the placenta, pancreatic islets, synovial tissues and circulating blood Like many proinflammatoy cytokines such as TNF-α, resistin targets adipocytes and enhances inflammation in adipose tissue through a distinct signaling pathway despite that factor that both of them activates NF-κB. Several studies suggest that increasing levels of resistin is involved in insulin resistance and type 2 diabetes (Filkova et al., 2009; Kusminski et al., 2005). A recent study has shown that resistin induced β-cell apoptosis in rat RINm5F insulinoma cells (Gao et al., 2009). In several cell lines, Akt, a downstream target of PI3K, can be phosphorylated by resistin which leads to attenuation of insulin signaling (Gao et al., 2007a).

1.2.3.1.3 Inflammation in obesity

Obesity is associated with a state of chronic, low-grade systemic inflammation. There is now much evidence that a strong relation exists between distribution and metabolism of adipose tissue and inflammation. Visceral adipose tissue is reported to be more metabolically active and has a higher lipolytic rate than subcutaneous adipose tissue. Several studies have demonstrated the association between visceral adiposity and several inflammatory markers such as C-reactive protein (CRP), TNF-α, IL-6, macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein (MIP). The mechanism of cytokine production by the adipose tissue has been demonstrated by several studies. In summary, the adipose tissue undergoes a constant remodeling that is accelerated in the case of obesity. Accumulation of fat in adipocytes induces hypertrophy and increases endoplasmic reticulum stress and number of dead adipocytes. This process is followed by infiltration of immune cells such as macrophages, T-lymphocytes and neutrophils. Throughout cross talk between macrophages and the adipocytes, inflammatory cytokine and leptin production is enhanced. On the other hand, the production of the anti-inflammatory adipokine adiponectin is downregulated (Lee et al., 2010). Leptin receptors have reported on the surface of various immune cells and have been associated with production of TNFα- and IL-6. The macrophage derived TNF-α enhances the release of FFAs via lipolysis. FFAs and

inflammatory cytokines in their turn drain into the portal circulation, where they can substantially affect glucose and lipid metabolism in the liver. Moreover, FFAs are a potent ligand of Toll-like receptors on the macrophages. These receptors mediate activation of NF-κB pathway; hence a vicious circle of inflammation that involves adipocyets and marcophages is initiated (Figure 4) (Mathieu et al.; Suganami and Ogawa, 2010).

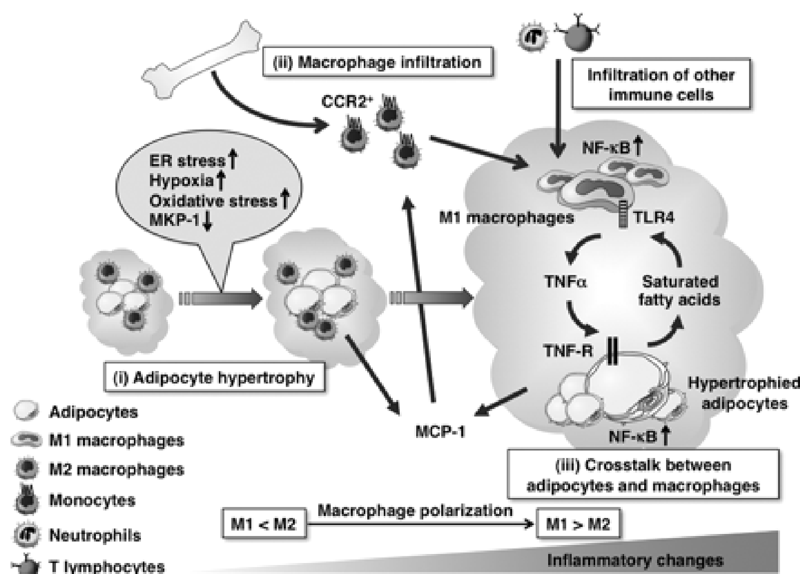

Figure 4 Molecular mechanism underlying adipose tissue inflammation (Suganami and Ogawa, 2010)

It has been suggested that the inflammatory state of obesity and particularly the production of inflammatory adipokines, is associated with complications of obesity such as type 2 diabetes, as well as the other components of the metabolic syndrome (Clement and Langin, 2007). It is also noteworthy that insulin resistance is established, in part, through the phosphorylation of IRS on serine residues by inflammatory cytokines such as TNF-α (Hotamisligil, 1999).

1.2.3.2 Pancreatic Beta Cell dysfunction

Normal beta cells exhibit a biphasic insulin secretion in response to a rapid and sustained glucose challenge. The first phase of insulin response is a transient phase that peaks in 5-10 minutes, and decays toward baseline in 20 minutes. During this phase, the

liver responds quickly by switching off glucose production. A second sustained phase has a lower peak, is often oscillatory in nature and can last for several hours. A defect in the first-phase insulin response was detected in subjects with impaired glucose tolerance (IGT) and in the early stage of type 2 diabetes (Saltiel and Kahn, 2001). A defective second-phase insulin secretion detected in individuals with T2DM is the manifestation of complete β-cell failure and this necessitates insulin therapy (Gerich, 2002).

Several mechanisms underline the progressive loss of β-cell function. Some studies suggest that increased islet apoptosis and diminished proliferation are genetically programmed. Chronic hyperglycemia, hyperlipidemia and deposition of islet amyloid are thought to be involved in reduction of β-cells mass and ultimately β-cell failure (Steppel and Horton, 2004), (Hoppener et al., 2000).

1.2.4 Diabetes complications

Diabetes mellitus is clearly associated with a number of long-term microvascular and macrovascular complications. The etiology of these complications is likely multifactorial and closely related to chronic hyperglycemia. The microangiopathies of diabetes include retinopathy, nephropathy, and peripheral and autonomous nervous system damages. Although microangiopathies represent a serious problem in diabetic patients, mortality and morbidity rates in type 2 diabetes are often associated with macroangiopathic complications such as coronary artery disease, peripheral vascular disease and cerebrovascular disease (Singhania et al., 2008).

1.2.5 Treatment

1.2.5.1 Lifestyle interventions

The majority of cases of type 2 diabetes can be avoided by lifestyle modification. A combination of factors such as weight loss, regular exercise, eating a diet high in fiber and low in saturated and trans-fats, smoking cessation and alcohol reduction was associated with a reduced risk for type 2 diabetes (Klein et al., 2004).

1.2.5.2 Pharmacological treatment

Some patients with type 2 diabetes mellitus respond well to body weight reduction, diet and exercise. However, most patients eventually require drug therapy to maintain adequate glycemic control, in part because adherence to lifestyle changes is difficult to maintain or integrate. Several classes of oral antidiabetic drugs are currently available but due to the progressive nature of type 2 diabetes, a considerable proportion of patients will eventually resort to insulin therapy. In these patients, insulin is used either as monotherapy or as combined therapy with other oral antidiabetic agents, to maintain adequate glycemic control (Ambavane et al., 2002), (Krentz and Bailey, 2005).

The main classes of oral antidiabetic drugs include those that increase insulin secretion, known as insulin secretagogues (sulphonylureas and rapid-acting secretagogues), insulin sensitizers (biguanides and thiazolidinediones), inhibitors of intestinal carbohydrate digestion and absorption (α-glucosidase inhibitors) (Krentz and Bailey, 2005) and the novel class of incretin-based antidiabetic drugs (Ng et al., 2007).

1.2.5.2.1 Insulin secretagogues

1.2.5.2.1.1 Sulfonylureas

These drugs lower blood glucose concentrations by stimulating insulin secretion by binding to the β-cell's sulphonylurea receptor (SUR-1), which is a member of the ATP-binding cassette or the traffic ATPase superfamily. This interaction inhibits the conductance of adenosine triphosphate (ATP)- dependent postassium (K_{ATP}) channels leading membrane depolarisation and opening of voltage-dependent calcium channels. The rise in the intracellular calcium concentration leads to increased fusion of insulin containing granules and insulin exocytosis (Ducobu, 2003).

The main adverse effect of sulfonylureas is hypoglycaemia, which can be prolonged and life-threatening, although rarely. The first generation sulfonyureas such as chlorpropamide and glibenclamide are associated with a greater risk of hypoglycaemia than the second-generation sulfonylureas (gliclazide, glimepiride, glipizide) because they bind carrier proteins in the blood which makes them prone to drug interaction.

Sulfonylureas can induce significant weight gain, mainly as a result of increasing insulin levels (Krentz and Bailey, 2005).

1.2.5.2.1.2 Rapid-acting insulin secretagogues (the glitinides)

Glitinides stimulate rapid, but short-term insulin secretion, thus should be taken immediately before meals to improve postprandial glycemic control. Glitinides include the carbamoylmethyl benzoic acid derivative repaglinide and the d-phenylalanine derivative nateglinide (Ambavane et al., 2002). Like sulphonylureas, these agents bind to the SUR1 in the plasma membrane of the β-cell but at a different binding site. They provide superior glycemic control when used in combination therapy with metformin or thiazolidinediones. Due to their short metabolic half-life, hypoglycaemia, if ever occurs, is rarely prolonged (Schmitz et al., 2002), (Dailey, 2005).

1.2.5.2.2 Insulin sensitizers

1.2.5.2.2.1 Biguanides

Metformin is the only biguanide currently available on the market after the withdrawal of phenformin from the United States in 1975 due to induction of lactic acidosis. It ameliorates hyperglycemia without increasing insulin secretion, weight gain, or hypoglycaemia. This drug has been shown to have several metabolic effects. However, despite numerous studies on metformin, its complete cellular mechanisms of action have yet to be fully identified. The major mechanism of action of metformin is to suppress hepatic glucose production by improving hepatic sensitivity to insulin on the one hand and decreasing the availability of certain gluconeogenic substrates (e.g. lactate) on the other hand. Metformin also enhances peripheral insulin sensitivity; it increases insulin-dependent glucose uptake by skeletal muscle through a mechanism involving GLUT4 translocation to the cell membrane. Moreover, metformin acts in an insulin-independent manner to induce fatty acids oxidation, suppress lipogenic enzymes expression and reduce triglyceride levels in patients with hypertriglyceridaemia. Until now, these pleiotropic therapeutic benefits of this drug are almost all attributed to activation of AMPK (Zhou et al., 2001), (Krentz and Bailey, 2005).

1.2.5.2.2.2 Thiazolidinediones (TZDs)

Agents of this class are selective ligands of the nuclear transcription factor peroxisome-proliferator–activated receptor γ (PPARγ). The PPAR receptors (PPARs) belong to a subfamily of the nuclear-receptor superfamily that regulates gene expression in response to ligand binding. Since their initial discovery, three PPARs have been identified, namely PPARα, PPARδ/β and PPARγ. Troglitazone was the first drug of this class to be available but was later withdrawn due to idiosyncratic hepatic failure. Two other TZDs, rosiglitazone and pioglitazone have been approved for use as antidiabetic medication by USA Food and Drug Administration (FDA). These two drugs have notably been shown to be devoid of hepatotoxic potential (Norris et al., 2007).

Several mechanisms can explain the improvement of insulin sensitivity by TZDs. First, stimulation of PPARγ by TZDs promotes differentiation of pre-adipocytes to adipocytes with accompanying fat storage and reduction of FFAs. This leads to enhanced insulin signaling in insulin-sensitive tissues. Second, PPARγ activation by TZDs inhibits secretion of pro-inflammatory cytokines such as TNFα, and increases adiponectin levels (Yki-Jarvinen, 2004). Finally, TZDs have been shown to activate AMPK in liver, skeletal muscle and adipocytes (Fryer et al., 2002), (Hardie et al., 2006).

1.2.5.2.3 α-Glucosidase Inhibitors

Drugs belonging to this class, such as acarbose and miglitol, competitively and reversibly inhibit the activity of α-glucosidase enzymes in the intestinal brush border membrane. The α-glucosidase enzyme is responsible for poly- and disaccharide digestion and release of monosaccharide. Therefore, these inhibitors should be taken with meals containing digestible carbohydrates but not monosaccharides as they do not affect the absorption of glucose. Gastrointestinal problems including nausea, vomiting, flatulence and diarrhea constitute the main side-effects of α-glucosidase Inhibitors treatment (Toeller, 1994), (Krentz and Bailey, 2005).

1.2.5.2.4 Dipeptidyl peptidase 4 inhibitors and GLP-1 analogues

Incretins are gastrointestinal hormones that enhance absorbed glucose disposal through the stimulation of insulin release and the inhibition of glucagon secretion. The two major incretin hormones are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Incretins are rapidly degraded to inactive form by the enzyme dipeptidylpeptidase 4 (DPP-4), hence their therapeutic utility is very limited (Verspohl, 2009). Therefore, two current therapeutic approaches have been developed to enhance the endogenous incretin action: incretin mimetics resistant to DPP-4 degradation such as the GLP-1 analogues exenatide and liraglutide, and the DPP-4 inhibitors such as sitagliptin and vildagliptin (Halimi, 2008). Several other compounds are under clinical development.

1.2.5.3 Herbal medicine and diabetes

Despite all the strategies and treatments used to manage type 2 diabetes, the overall glycemic control in diabetic patients remains unsatisfactory. As a result, many people all over the world are gradually reverting to the use of complementary and alternative medicine (CAM). In general, 38% of adults at the age of 18 years or older use CAM in the United States according to 2007 National Health Interview Survey (NHIS) with the natural products as being the most used CAM therapy (17.7% of adults using CAM). Individuals with life threatening diseases, such as cancer and HIV, are more likely to use CAM (Barnes et al., 2008). In Canada, one third of type 1 and type 2 diabetes patients attending diabetes education programs were reported to use CAM, including herbal remedies (Nahas and Moher, 2009).

The use of herbal remedies to treat diabetes in several parts of the world can be traced back thousands of years ago. Medicinal plants have been incorporated into the traditional medicine of many ancient civilizations such as those of China, India, Egypt and Greece. Moreover, many modern pharmaceuticals are plant-derived. An example is metformin, which is derived from the naturally occurring compound guanidine. The later is

isolated from *Galega officinalis*, also known as French Lilac or goat's rue, a common traditional remedy for diabetes (Yeh 2003).

Coccinia indica (ivy gourd), *Ocimum sanctum* (holy basil) and *Gymnema sylvestre* are used to treat diabetes described as "sugar urine" (madhumeha) in Ayurveda, an ancient Indian healing system. *Trigonella foenum graecum* (fenugreek) is widely used in herbal medicine in North Africa, India and the Middle East (Haddad et al., 2001). In early Greek and Latin pharmacopoeias, defatted fenugreek seeds have been recommended for diabetes. *Bauhinia forficate*, referred to as "vegetable insulin", and *Myrcia uniflora* have been used for treatment of diabetes in Brazilian and South American herbal medicine. *Ficus carica* (fig leaf) is a popular herbal treatment for diabetes in Spain and South-western Europe. *Opuntia streptacantha* (nopal) or the prickly pear cactus is native to southeast USA and is commonly used to treat diabetes among Mexican Americans (Yeh et al., 2003). *Momordica charantia*, also known as bitter melon, has been used for a long time to treat diabetes in Asia, Africa, India and South America. In fact, several constituents of this plant are found to have antidiabetic properties, among these are the two steroid glycosides charantin, vicine, as well as polypeptide-p (an insulin-like protein) (Basch et al., 2003), (Yeh et al., 2003).

1.3 Genus *Vaccinium*

The genus *Vaccinium* belongs to the family Ericaceae and consists of more than 150 species. Some plants of this genus bear edible fruits that have attracted the attention of the industry for the production of juices and jams (Hjalmarsson and Ortiz, 2001).

Various members of this genus have been used in traditional medicine to treat symptoms related to diabetes (Cignarella et al., 1996), (Chambers and Camire, 2003), (Leduc et al., 2006). Leaves and fruits of *V. myrtillus* L (European blueberry or bilberry) were the most widely used antidiabetic preparation in Europe prior to the discovery of insulin (Helmstadter, 2007). The commercial drug Difrarel®, prescribed for circulatory disorders, contains 100 mg of bilberry anthocyanins (Meskin, 2002).The infusion of *V.*

ashei reade (rabbiteye blueberry) leaves has been used as a folk medicine in Europe for the treatment of lifestyle-related diseases (Sakaida et al., 2007).

Consumption of unsweetened cranberry juice (*V. macrocarpon* Ait.) improved glycemic control in patients with type 2 diabetes (Wilson et al., 2008). *V. angustofolium* Ait. (Canadian lowbush blueberry) is highly recommended by Quebec traditional practitioners (Haddad et al., 2001) and is also mentioned by Cree Elders of Eeyou Istchee for treatment of diabetic symptoms and complications, (Leduc et al., 2006), (Martineau et al., 2006). A recent study has shown that the biotransformed juice of *V. angustifolium* incorporated in the drinking water of diabetic KKA^y mice lowered blood glucose levels (Vuong et al., 2009).

1.3.1 *Vaccinium vitis-idaea*

V. vitis is an evergreen dwarf shrub that grows widely in northern temperate, boreal and subarctic zones and is particularly used in Scandinavian culture as herbal medicine and in the preparation of traditional meals. The flowers of this plant are produced singly or in cluster of up to 15 and have white to pinkish red, bell-shaped corolla (Figure 2). The berries are bright red and globular, approximately 0.75 to 1.0 cm in diameter (Figure 2). Interestingly, the berries of this plant were stored without the use of sugar due to their high content of benzoic acid (Wang et al., 2005), (Hjalmarsson and Ortiz, 2001), (Stang et al., 1990). *V. vitis* berry is an economically important crop and has been used to produce juice, sauce, candy, jelly, syrup, ice cream, pickle and liqueur (Stang et al., 1990).

V. vitis has around 25 common names worldwide. The mostly used English names are lingonberry, cowberry, moss cranberry, mountain cranberry, partridgeberry, red whortleberry, alpine cranberry and lingon or lingen (Stang et al., 1990), (Cheng et al., 2005). Hultén identified two sub-species of *V. vitis*: the North American *V. vitis-idaea* ssp. minus (Lodd.) Hult and the larger European plant as the variety *vitis-idaea* L. The two subspecies can be distinguished mainly by their size, *V. vitis-idaea* measures more than 30 cm, whereas *V. vitis-idaea* ssp. minus rarely exceeds 20 cm (Stang et al., 1990), (Hjalmarsson and Ortiz, 2001), (Gustavsson, 2001).

Since ancient times, the stem and leaf of *V. vitis* have been used as an anti-inflammatory folk medicine to treat respiratory system infections in China (Wang et al., 2005). In Swedish folk medicine, *V. vitis* drink has been used to treat fever, diarrhea and scurvy. Tea, derived from leaves, has been promoted as a cure to urinary tract infections and as anti-rheumatic medication (Hjalmarsson and Ortiz 2001). These effect were confirmed by recent studies where the leaves and berries of this plant were shown to have anti-microbial and anti-inflammatory effects (Fokina et al., 1993), (Tunon et al., 1995). In addition, other studies have shown that *V. vitis* berries had anticancer activity *in vitro* which was attributed to its high anthocyanin content. Finally, *V. vitis* showed a higher antioxidant activity as compared to other berries including blackberries, blueberries and cranberries (Wang et al., 2005).

Figure 5 Flowers and fruits of *V. vitis*-idaea

1.3.1.1 Phytochemicals of *V. vitis*

In addition to vitamins C and E, and carotenoids, *V. vitis* contains a wide range of bioactive compounds that belong mainly to flavonoid, phenolic acid, tannin and stilbene classes (Perry 1980), (Wang et al., 2005), (Rimando et al., 2004), (Kahkonen et al., 2001).

1.3.1.1.1 Flavonoids

Flavonoids constitute the largest and most important group of polyphenolic plant secondary metabolites with more than 6000 known compounds (Pietta, 2000), (Schijlen et

al., 2004). Flavonoids have attracted a lot of attention due to their health-promoting effects. Due to the presence of aromatic hydroxyl groups, they have strong antioxidant properties, where they act as scavengers against reactive oxygen and nitrogen species. Therefore, flavonoids protect DNA, proteins and lipoproteins as well as membrane lipids (Vessal et al., 2003), (Ciz et al., 2008). Among the different classes of flavonoids, anthocyanins, flavonols and flavan-3-ols are the main classes detected in *V. vitis*

1.3.1.1.1.1 Anthocyanins

Anthocyanins are the most important water-soluble pigments in plants, producing blue, red and purple colours. They exist as glycosides and acylglycosides of the aglycone anthocyanidins. The most common sugar constituents found in these compounds are glucose, rhamnose, xylose, galactose, arabinose, and fructose (Wang et al., 1997). Anthocyanins from blueberries were reported to have a remarkable antidiabetic activity in vivo (51% reduction in blood glucose levels) using diabetic C57bl/6J mice (Grace et al., 2009). Cyanidin-3-glucoside, cyanidin-3-galactoside and cyanidin-3-arabinoside were identified in the berries and the aerial parts of *V. vitis* naturally growing in Finland (Ek et al., 2006).

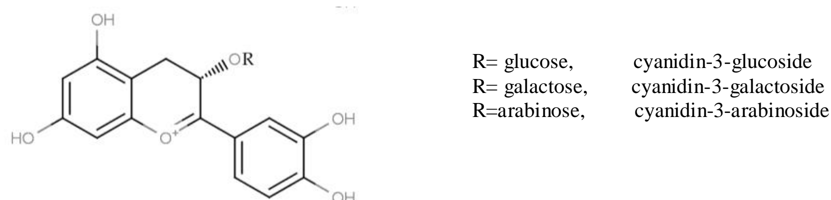

Figure 6 The structures of anthocyanidins detected in *V. vitis*-idaea

1.3.1.1.1.2 Flavonols

Quercetin and kaempferol along with their glycosides are the most common flavonols in *V. vitis* berries, although, quercetin glycosides are more abundant than kaemferol ones (Hokkanen et al., 2009). It was reported that Kaempferol and quercetin improved insulin-stimulated glucose uptake in mature 3T3-L1 adipocytes (Fang et al., 2008). In addition, quercetin exhibited antidiabetic properties in streptozocin-induced diabetic animals (Vessal et al., 2003), (Shetty et al., 2004b).

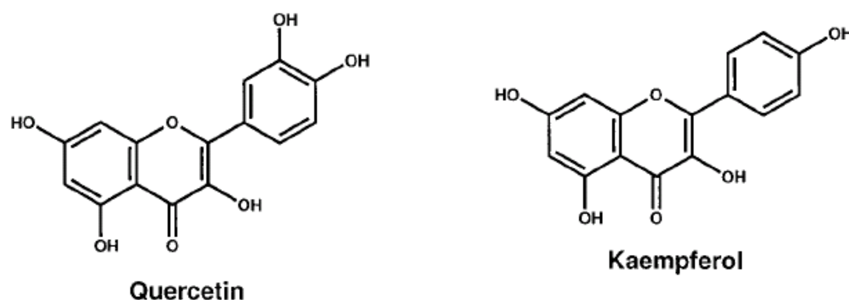

Figure 7 The structures of quercetin and kaempherol

1.3.1.1.1.3 Flavan-3-ols

Flavan-3-ols (catechins and their polymers proanthocyanidins) are widely distributed in the plant kingdom. Catechins constitute one-fifth of the total estimated intake of flavonoids (Kuhnau, 1976), (Einbond et al., 2004). (+)-Catechin and (−)-epicatechin are the predominant catechins in *Vaccinium* species. The content of catechins was found to be 10 times higher in *V. vitis* (250 mg/kg fresh weight) than in the other *Vaccinium* species (cranberry, 30 mg/ kg and bilberry, 75 mg/kg) (Maatta-Riihinen et al., 2004). Proanthocyanidins will be discussed in more detail under the class of tannins.

Previous studies have demonstrated that (−)-epicatechin, normalized blood glucose levels, protected normal rat β-cells and promoted β-cell regeneration in islets of alloxan-treated rats (Chakravarthy et al., 1981), (Chakravarthy et al., 1982).

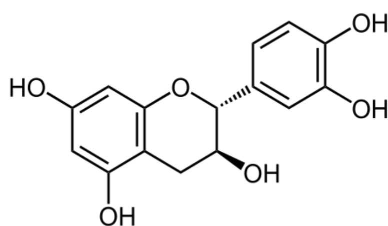

Figure 8 The structure of catechin

1.3.1.1.2 Phenolic acids

Phenolic acids and their esters, amides and glycosides are also important constituents of our diet. Two classes of phenolic acids can be distinguished: derivatives of benzoic acid and derivatives of hydroxycinnamic acid (Manach et al., 2005). Indeed, the

berries of *V. vitis* have high content of free benzoic acid but not significant amount of its derivatives (Visti et al., 2003).

On the other hand, chlorogenic acid (ester of caffeic acid) is the major hydroxycinnamic acid derivative and the greatest contributor of total polyphenolic content in highbush blueberries (Lee et al., 2002). Caffeic acid, ferulic acid and coumaric acid glycosides are the most predominant hydroxycinnamic acids in berries and leaves of *V. vitis* (Ek et al., 2006)

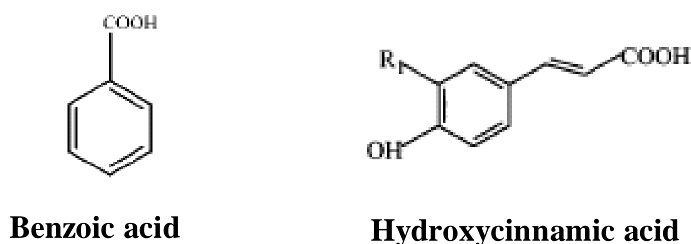

Figure 9 The structures of benzoic acid and hydroxycinnamic acid

1.3.1.1.3 Stilbenes

This group of phenolics is present in considerable quantities in grapes and wine (Rimando et al., 2004). Stilbenes have been reported to possess anticancer and antioxidant activities (Rimando et al., 2005).

Resveratrol was found in all *Vaccinium* species, *V. vitis* obtained from Nova Scotia, Canada, contains the highest concentration of resveratrol, almost the same as that found in grapes (6500 ng/g dry sample) (Rimando et al., 2004). Resveratrol is well known for its cardioprotective effect (Das and Maulik, 2006). However, recent *in vitro* and *in vivo* studies indicate that resveratrol has also anti-diabetic properties (Breen et al., 2008), (Szkudelska and Szkudelski), (Su et al., 2006), (Chi et al., 2007), (Penumathsa et al., 2008).

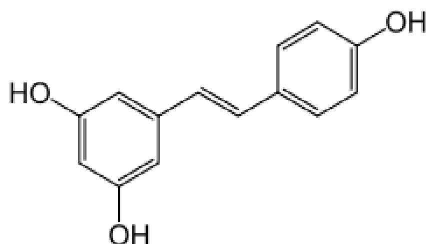

Figure 10 The structure of resveratrol

1.3.1.1.4 Tannins

Tannins are water-soluble polyphenols that are present in many plants and have a molecular weight between 500 and 3000 Da (Haslam, 1988). There are two classes of tannins: hydrolyzable and nonhydrolyzable (condensed) tannins. Hydrolyzable tannins are composed of a central core of a polyhydric alcohol such as glucose, in which the hydroxyl groups are partially or totally esterified by either gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) (Chung et al., 1998).

Condensed tannins are structurally more complex than hydrolyzable tannins. They are the polymers of flavan-3-ols and flavan-3,4-diols, or a mixture of the two (Chung et al., 1998). Oligomers and polymers of catechins linked principally through the C-4 of the flavanol unit are called proanthocyanidins or procyanidins (Duenas et al., 2003). The structures of procyanidins isolated from berries of *V. vitis* are shown in figure 8 (Ho et al., 2001). Proanthocyanidin A was also detected in berries and leaves of *V. vitis* (Ek et al., 2006).

The biological activities of tannins include anti-oxidant, anti-tumoural, anti-microbial, anti-HIV, platelet aggregation inhibitory, antidiarrhoeal and anti-inflammatory activities (Yokozawa et al., 1993), (Chung et al., 1998), (De Bruyne et al., 1999).

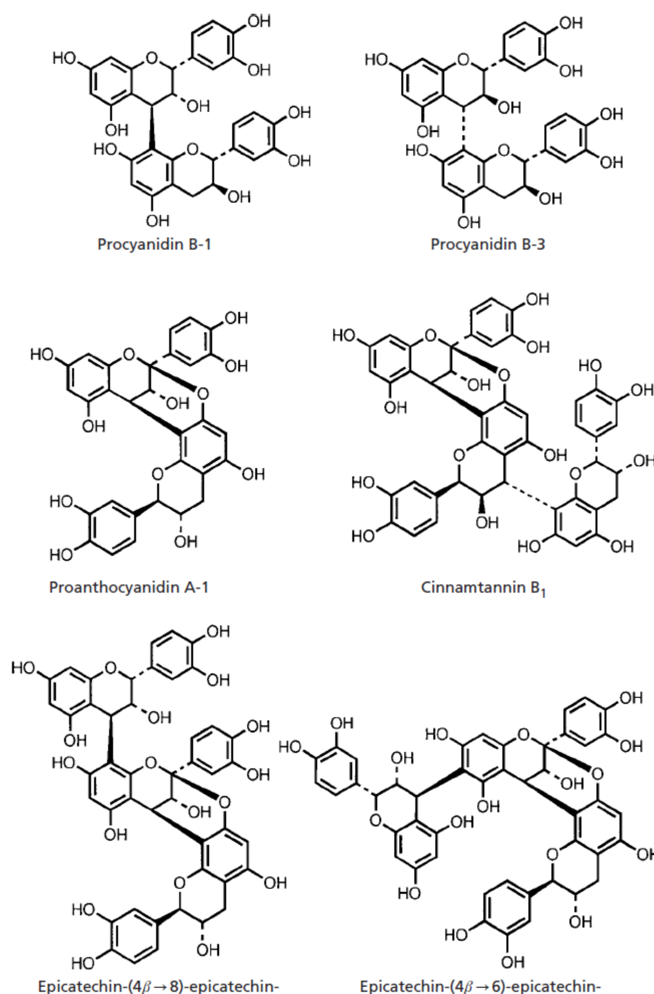

Figure 11 Structure of tannins isolated from *V. vitis*-idaea (Ho et al., 2001)

1.4 Scope and objectives of the study

V. vitis was identified by our team through the ethnobotanical survey carried out in Whapmagoostui First Nation (Fraser et al., 2007). In a subsequent bioassay screening study, *V. vitis* emerged as the most promising plant with antidiabetic activity since it increased basal and insulin-dependent glucose uptake in murine C2C12 skeletal and 3T3 adipocyte cell lines (Harbilas et al., 2009). The aim of this thesis is to investigate the mechanisms of the antihyperglycemic activity of the crude extract of *V. vitis*. With the help of bioactivity-guided fractionation and different chromatographic methods, the active compounds of this extract will be identified and their structure will be elucidated. The pure compounds will be then evaluated for the antidiabetic activity and the involvement of the different signaling pathways that regulate glucose metabolism will be determined. The

final goal of this study is to conduct validation studies in order to confirm the *in vivo* antidiabetic activity of *V. vitis* in animal models of diabetes.

In the course of the fractionation procedure, a caffeic acid derivative was identified and later understood to represent an artefact of the interaction of the eluting solvent with *V. vitis* components. Given the outstanding potency of this compound in stimulating skeletal muscle cell glucose uptake, a structure-activity study was carried out.

2. Article 1

Stimulation of AMPK and enhancement of basal glucose uptake in muscle cells by quercetin and quercetin glycosides, active principles of the antidiabetic medicinal plant *Vaccinium vitis-idaea*

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Authors' contribution

I performed the experimental work, data analysis and wrote the paper.

Louis Martineau contributed to the elaboration of protocols, interpretation of data and correction of the paper.

Ammar Saleem and Asim Muhammad helped with the isolation, the identification and the quantification of *V. vitis* active constituents.

Ali Benhaddou Andaloussi and Lidia Nistor contributed to the elaboration of glucose uptake protocol.

Diane Vallerand performed the mitochondrial respiration assay.
Arvind Afshar performed the cell culture rate of acidification assay as well as the cytosolic ATP assay for quercetin.
Dr Pierre Haddad is my supervisor.

Running title

Antidiabetic mechanism of *Vaccinium vitis-idaea*

Keywords: Aboriginal populations of North America • Bioassay-guided fractionation • Flavonoids • Mitochondria • Traditional medicine

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Abstract

Several medicinal plants that stimulate glucose uptake in skeletal muscle cells were identified from among species used by the Cree of Eeyou Istchee of northern Quebec to treat symptoms of diabetes. This study aimed to elucidate the mechanism of action of one of these products, the berries of *Vaccinium vitis idaea*, as well as to isolate and identify its active constituents using a classical bioassay-guided fractionation approach. Western immunoblot analysis in C2C12 muscle cells revealed that the ethanol extract of the berries stimulated the insulin-independent AMP-activated protein kinase (AMPK) pathway. The extract mildly inhibited ADP-stimulated oxygen consumption in isolated mitochondria, an effect consistent with metabolic stress and the ensuing stimulation of AMPK. This mechanism is highly analogous to that of Metformin. Fractionation guided by glucose uptake activity resulted in the isolation of ten compounds. The two most active compounds, quercetin-3-*O*-glycosides, enhanced glucose uptake by 38-59% (50 µM; 18 h treatment) in the absence of insulin. Quercetin aglycone, a minor constituent, stimulated uptake by 37%. The quercetin glycosides and the aglycone stimulated the AMPK pathway at concentrations of 25-100 µM, but only the aglycone inhibited ATP synthase in isolated mitochondria (by 34 and 79% at 25 and 100 µM, respectively). This discrepancy suggests that the activity of the glycosides may require hydrolysis to the aglycone form. These findings indicate that quercetin and quercetin 3-*O*-glycosides are responsible for the antidiabetic activity of *V. vitis* crude berry extract mediated by AMPK. These common plant products may thus have potential applications for the prevention and treatment of insulin resistance and other metabolic diseases.

Introduction

Aboriginal populations allover world are particularly at risk for developing type II diabetes mellitus. The same genetic attributes that have favored the survival of these populations in harsh environments have now turned into a liability, increasing susceptibility to metabolic diseases when a sedentary lifestyle and a calorie-dense diet are adopted [1][2]. The incidence rate of diabetes in these populations is often accompanied by a disproportionately high rate of diabetic complications, including nephropathy, retinopathy, and peripheral neuropathy, a phenomenon attributed to low adherence to modern anti-diabetic medications [1][3]. As these complications more than diabetes itself contribute to a decrease in quality of life and to important social costs, there is an imperative to develop treatment options that are well-adapted from a cultural perspective in order to ensure adherence.

One approach is to identify efficacious treatments for diabetes within the traditional pharmacopea of the affected populations and to promote the integration of such products into the diet. This is the approach that our research team has been using in order to address this issue in Canadian native populations, specifically the Cree of Eeyou Istchee (Northeastern James Bay area of the Canadian province of Quebec), a population experiencing one of the highest rates of diabetes in Canada [4-9]. In collaboration with the Cree of Eeyou Istchee, we have conducted two ethnobotanical surveys and identified 17 medicinal plant species that are traditionally used to treat symptoms related to diabetes [10][11]. Two bioactivity-screening projects for antidiabetic properties using cell-based assays have revealed that over half of the 17 species enhance glucose uptake in skeletal muscle cells [12][13]. Seven species identified through a survey of the community of Mistissini [10] and found to promote glucose uptake [12] were recently studied together in an attempt to elucidate their mode of action [14]. This study concluded that, in all cases, activity involved the AMP-activated protein kinase (AMPK) pathway, a well-recognized therapeutic target for metabolic diseases and mediator of the effects of Metformin [15][16]. Moreover, the activation of AMPK was related to a transient disruption of mitochondrial energy tranduction, a mechanism analogous to that of Metformin [17]. Although the active

principles were not identified, such effects on mitochondrial function were observed to be consistent with the anti-microbial role of many plant metabolites [18].

This study focuses on the glucose-uptake-enhancing effects of the berries of *Vaccinium vitis idaea*, also known as Mountain cranberry or lingonberry, a medicinal plant product used in the communities of Whapmagoostui and Mistissini to treat frequent urination and a number of other symptoms of diabetes [10]. This product was the most active to emerge from our second bioactivity-screening project [13]. Various members of the *Vaccinium* genus, including lowbush blueberry (*V. angustifolium*), American cranberry (*V. macrocarpon*) and European bilberry (*V. myrtillus*), are traditionally used for the treatment of diabetes by several cultures throughout the world [19]. The goal of this study was to test the hypothesis that the enhancement of glucose uptake by *V. vitis idaea* berry extract is mediated by a mechanism similar to that of the boreal forest medicinal plant species studied previously [14] and to simultaneously elucidate the active principles of this medicinal species using our expertise in the phytochemistry of *Ericacea* [20][21]. We conclude that quercetin and certain glycosides of this well-studied and widely distributed flavonoid [22] transiently inhibit mitochondrial ATPsynthase, leading to the activation of AMPK, and propose that quercetin and quercetin glycosides are responsible for the antidiabetic activity of *V. vitis* and perhaps of other species of this genus.

Materials and Methods

Plant material and extraction

Berries of *V. vitis idaea* L. (*V. vitis*) were collected in Whapmagoostui, Que., Canada, and kept at -20°C until use. Plant material was authenticated by a taxonomist (A. Cuerrier, Montreal Botanical Garden, Montreal, Que., Canada) and voucher specimens were deposited at the Montreal Botanical Garden herbarium (voucher Whap04-21). In total 800 g of the berries were freeze-dried (Super Moduylo freeze dryer; Thermo Fisher, Ottawa, Ont., Canada) to yield 114 g of dry material. The dry material was then extracted three times for 24 h with ten volumes of 80% ethanol on a mechanical shaker and then filtered under vacuum using Whatman 1 paper. The supernatants were combined and dried using a rotary evaporator (RE 500; Yamato Scientific, Tokyo, Japan) followed by lyophilization. Preliminary phytochemical characterization of *V. vitis* berry crude extract in the form of extract yield, total phenolic content and identification of a small number of marker compounds, have been reported in an earlier study [13]. Markers include catechin, para-coumaric acid, cyanidin glycosides, and quercetin glycosides. The freeze-dried ethanol extract was reconstituted in water (15% w/v) and extracted in a separatory funnel with equal volume of ethyl acetate to yield an ethyl acetate soluble fraction. The aqueous solution remaining after ethyl acetate extraction was freeze-dried and kept for bioactivity screening. Crude extract and fractions were solubilized in DMSO at 200 mg/mL, aliquoted, and stored at -20°C until bioactivity testing. Isolates were similarly prepared and used at a final concentration of 100 mM. Quercetin and quercetin-3-*O*-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin-3-*O*-galactoside was purchased from Indofine Chemical (Hillsborough, NJ, USA). Pure compounds were reconstituted to a concentration of 100 mM in DMSO, aliquoted, and stored frozen.

Cell culture

C2C12 murine skeletal myoblasts and H4IIE murine hepatocytes were obtained from the American Type Cell Collection (ATCC; Manassas, VA). Cell culture media were purchased from Invitrogen Life Technologies (Burlington, ON) unless otherwise noted.

Other reagents were purchased from Sigma–Aldrich (Oakville, ON) unless otherwise noted. C2C12 myoblasts were cultured in 6- or 12-well plates in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 % horse serum (HS) and antibiotics (penicillin 100 U /mL, streptomycin 100 µg /mL) at 37 °C in a 5 % CO_2 atmosphere. After 80 % confluence, myoblasts were differentiated into myotubes in DMEM supplemented with 2 % HS and antibiotics for exactly 7 days, resulting in the fusion of all cells into multinucleated myotubes. H4IIE hepatocytes were grown in 6-well plates DMEM supplemented with 10 % FBS until fully confluent and experiments were performed 1 to 3 days later. Treatments were initiated 18 h prior to glucose uptake or signaling experiments. Aliquots of crude extract and fractions were diluted in differentiation medium at 1:1000 for a final DMSO concentration of 0.1 % and a final extract or fraction concentration of 200 µg /mL. The crude extract concentration of 200 µg /mL was previously used for bioactivity screening and determined to be non-cytotoxic [13]. Aliquots of isolates or pure compounds were diluted in differentiation medium at 1:1000 for a final concentration of 100 µM. To obtain, concentrations of 50 and 25 µM, original aliquots were diluted at 1:2000 and 1:4000, respectively, and DMSO was added to maintain final concentration at 0.1 % in all conditions.

Glucose uptake assay

The effects of plant products on the rate of uptake of glucose by differentiated C2C12 skeletal myotubes were assessed with a ^{3}H-deoxyglucose uptake assay as described previously [12][13][23][24]. Briefly, treatments or vehicle alone were applied for 18 h to 6-day differentiated cells. Following the treatment period, cells were rinsed twice with Krebs-phosphate buffer (KPB; 20 mM HEPES, 4.05 mM Na_2HPO_4, 0.95 mM NaH_2PO_4, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 mM glucose) at 37°C and allowed to equilibrate in this buffer for 30 min at 37°C. During this time, insulin (100 nM) was added to some wells. Following this, cells were washed twice with glucose-free KPB at 37°C, and 0.5 µ Ci/mL 2-deoxy-D-[1-^{3}H]-glucose (TRK-383, Amersham Biosciences, Buckinghamshire, UK) in this same buffer was applied for exactly 10 min at

37°C. Cells were then placed on ice and rapidly washed three times with ice-cold KPB, and lysed with 0.1 M NaOH for 30 min. The lysate was added to 4 mL of liquid scintillation cocktail (Ready-Gel 586601; Beckman Coulter, Fullerton, CA, USA) and radioactivity was measured in a liquid scintillation counter (LKB Wallac 1219; Perkin-Elmer, Woodbridge, Ont., Canada).

Western immunoblot

The effects of plant products on the insulin and AMPK signaling pathways of C2C12 muscle cells or H4IIE hepatocytes were assessed by western immunoblot. Treatments or vehicle alone were applied for 18 h to 6-day differentiated C2C12 cells or to post-confluent H4IIE cells. Thirty minutes prior to the end of the treatment, insulin (100 nM) or aminoimidazole carboxamide ribonucleotide (AICAR; 1 mM) were added to some vehicle-treated wells as positive controls. Following treatment, cells were placed on ice and washed three times with ice-cold PBS (8.1 mM $NaHPO_4$, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) and lysed in 250 µL of lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholate, and 0.1% SDS) containing a commercial cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany) supplemented with 1 mM phenylmethanesulfonyl fluoride, and a cocktail of phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Lysates were scraped into microcentrifuge tubes, kept on ice for 30 min with periodical vortexing, then centrifuged at 600×*g* for 10 min. Supernatants were decanted and stored at -80°C until analysis. Protein content was determined by the bicinchoninic acid method (Thermo Scientific Pierce, Rockford, IL, USA) standardized to bovine serum albumin. Lysates were diluted to a concentration of 1.0 µg/µL total protein and boiled for 5 min in reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, and 0.01% bromophenol blue). Briefly, 100 µL of each sample were separated on 10% polyacrylamide full-size gels and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered

saline (20 mM Tris-HCl, pH 7.6, and 137 mM NaCl) containing 0.1% Tween-20. The blots were then incubated overnight at 4°C on a mechanical shaker in blocking buffer with phospho- or pan-specific antibodies against Akt or acetyl-coA carboxylase (ACC) at 1:1000 (Cell Signaling Technologies, Danvers, MA, USA). Membranes were washed five times with Tris-buffered saline Tween-20 followed by a 1.5 h incubation at ambient temperature with horseradish-peroxidase-conjugated secondary antibodies diluted 1:100 000 (Jackson Immunoresearch, Cedarlane Laboratories, Hornby, Ont., Canada). Revelation was performed using the enhanced chemiluminescence method and blue-light-sensitive film (Amersham Biosciences). Experiments were repeated on three different passages of cells, each passage containing all conditions in parallel. All samples from a given passage were separated and transferred simultaneously to a single membrane. Quantification of the integrated density of bands was performed using a flatbed scanner (ScanJet 6100; Hewlett Packard, Palo Alto, CA, USA) and NIH Image 1.63 software (National Institutes of Health, Bethesda, MD, USA).

Respiration of isolated liver mitochondria

The effects of the crude extract and of selected isolates on the function of mitochondria were assessed by oxygraphy. Mitochondria were isolated from the liver of male Wistar rats as *per* the method of Johnson and Lardy [25]. Surgery, isolation of mitochondria, and measurement of oxygen consumption were performed as described previously [26]. All animal manipulations were sanctioned by the animal ethics committee of the Université de Montréal and respected the guidelines from the Canadian Council for the Care and Protection of Animals. Briefly, rats obtained from Charles River (St. Constant, Que., Canada) and weighing between 200 and 225 g were anesthetized and laparotomized. The portal vein was cannulated and the hepatic artery and the infrahepatic inferior vena cava were ligated. The liver was flushed with 100 mL of Krebs-Henseleit buffer (25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, pH 7.4, 154 mM NaCl, 4.8 mM KCl, 2.1 mM $CaCl_2$, and 1.2 mM $MgSO_4$) at 22°C prior to excision. In total 2 g of tissue were homogenized on ice using a Teflon potter homogenizer in ice-cold isolation buffer (10 mM

Tris, pH 7.2, 250 mM sucrose, and 1 mM EGTA). The homogenate was centrifuged at 600×*g* for 10 min at 4°C in order to remove cellular fragments and the resulting supernatant was centrifuged at 12 000×*g* for 5 min at 4°C. The pellet was delicately washed once with this same buffer and re-centrifuged. The pellet was then washed once with EGTA-free buffer and again re-centrifuged. The final pellet, containing viable mitochondria, was suspended in EGTA-free isolation buffer and kept on ice. Protein content of the mitochondrial preparation was determined by Lowry protein assay. O_2 consumption was measured at 25°C in a Hansatech Oxygraph apparatus (Norfolk, UK) with a 1 mL reaction chamber, as described previously [26]. Briefly, 1 mg of mitochondrial protein was added to respiration buffer (5 mM KH_2PO_4, pH 7.2, 250 mM sucrose (ultra pure), 5 mM $MgCl_2$, 1 mM EGTA, and 2 µM of the complex I inhibitor rotenone) at 25°C in the reaction chamber, for a final volume of 990 µL. Mitochondrial respiration was initiated by the injection of 6 mM (final concentration) of the complex II substrate succinate, and the rate of basal oxygen consumption *per* milligram mitochondrial protein (the rate of basal oxygen consumption (RBOC) or state 4 respiration) was determined. In total 1 µL of 1000× concentrated plant extract or 1 µL of DMSO was then injected and its effect on RBOC was assessed. Basal respiration was allowed to proceed for at least 30 additional seconds. Oxidative phosphorylation (state 3 respiration) was induced by the addition of 200 µM (final concentration) ADP and the rate of ADP-stimulated O_2 consumption (RASOC) *per* milligram mitochondrial protein (RASOC) was determined. Extracts were tested in three different experimental sessions, with at least two replicate experiments *per* mitochondrial preparation. DMSO-vehicle control experiments were conducted at the beginning and end of each experimental session in order to establish the session-normal RBOC and RASOC and to ensure no loss in mitochondrial viability over the duration of the session, typically less than 4 h from the end of the isolation protocol. DMSO was confirmed to have no effect on the basal rate of O_2 consumption. The effect of each plant extract was evaluated as: (i) the increase in the RBOC (a measure of the magnitude of the uncoupling effect); (ii) the decrease in functional capacity (FC) *per* milligram protein (a measure of the magnitude of the uncoupling effect plus any additional inhibitory effect), where FC was defined as the difference of the RASOC (maximal

functional rate of consumption) and the RBOC (rate of consumption driven by proton leak and not contributing to ATP synthesis). Calculations were as follows: the average FC *per* milligram protein of the vehicle control experiments for a given session was calculated by subtracting the average RBOC from the average RASOC. For (i) above, the absolute increase in RBOC measured in a given experiment was expressed as a percentage of the average control FC for the session. For (ii) above, the FC measured in a given experiment was expressed as a percentage of the average control FC for the session to give the percentage residual FC.

Assay of cell culture rate of acidification

A spectrophotometric assay of change in cell culture medium pH over time was developed based on similar assays [27], [28]. The assay medium consisted of Dulbecco's PBS containing Phenol Red as a pH indicator and modified for reduced buffering capacity while keeping other ion concentrations within physiological range (modified Dulbecco's PBS (mD-PBS) 1.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 137 mM NaCl, 25 mM glucose, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, Phenol Red 0.1 mM, and deionized ultra-filtered water). This formulation resulted in a pH of 7.1, which was adjusted to 7.2 at ambient temperature with NaOH immediately prior to the assay using an Accumet pH meter with calomel electrode (Fisher Scientific). Absorbance of 100 μL samples of medium transferred to 96-well plates (Sarstedt, Montreal, Que., Canada) was measured at ambient temperature at 530 and 450 nm using a Wallac Victor 2 plate reader (Perkin-Elmer, St. Laurent, Que., Canada) and the ratio of A 530/A 450 was calculated. The relationship between pH and the log of this ratio was observed to be linear over the range of pH 6.4-7.2 (Fig. 1A) and was modeled with the following function: pH=0.765×ln (A 530/A 450) +7.61 (R^2=0.99). The buffering capacity of mD-PBS was determined to be linear and equal to 1.075 mM equivalents *per* pH units between pH 6.3 and 7.1. Experiments were performed on 7-day differentiated C2C12 muscle cells and on 1-day post-confluent H4IIE liver cells grown in 12-well plates. On the day of the experiment, cells were gently rinsed twice with mD-PBS, and then allowed to equilibrate in exactly 1.0 mL of mD-PBS for

30 min at 37°C in a humidified air atmosphere. The assay was started by gently mixing pre-warmed 3× concentrated treatments in a 500 µL volume of mD-PBS to the 1.0 mL volume of mD-PBS already present, for a final volume of exactly 1.5 mL and treatments at their final working concentration. After the rapid addition of treatments to all the wells of a single plate, an initial 100 µL sample of medium, corresponding to time 0, was transferred to microtiter plate for spectrophotometric analysis. Cells were then incubated at 37°C in a humidified air atmosphere for the duration of the experiment. At times 20, 40, 60, 120, 180, and 240 min, plates were stirred and a 100 µL sample of medium was transferred to a microtiter plate for analysis. Calculations of rate of acidification and cumulative secretion of acid equivalents over time accounted for the decreasing experimental volume with each sampling. As DMSO was observed to stimulate acidification, as noted by others [29], quercetin was solubilized in ethanol (final vehicle concentration of 0.08%). Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma-Aldrich) solubilized in ethanol was used at 5 µM as a positive control. Results were expressed as cumulative secretion of acid equivalents (micromoles) for four to five replicates *per* condition *per* time point.

Cytosolic ATP assay

Total cytosolic ATP was measured in cell lysates by luminescence using the ATPlite assay kit (Perkin-Elmer, Waltham, MA, USA), as *per* the manufacturer's protocol. Briefly, C2C12 myotubes in 24-well plates or H4IIE hepatocytes in 96-well plates were treated in parallel for 1, 3, or 6 h with extract or DMSO. FCCP was used at 5 µM as a positive control. Results were expressed in % ATP content of vehicle-treated wells for one to two experiments of three to four replicates per condition per time point.

Statistical analysis

Results are reported as means ± SEM, with the number of replicates and number of independent experiments indicated. Data were analyzed by one-way analysis of variance

with a Fisher post-hoc test or by t-test when appropriate using StatView software (SAS Institute, Cary, NC). Statistical significance was set at $p \leq 0.05$.

Fractionation, isolation, and identification

Fractionation of the ethyl acetate soluble fraction of *V. vitis* berry ethanol extract is shown in Fig. 1. Gel filtration chromatography of the ethyl acetate soluble fraction was performed using Sephadex LH-20 (Pharmacia, Uppsala, Sweden) as a stationary phase. Sephadex LH-20 (110 g) was soaked in methanol and loaded on a glass column (25×105 cm). The mobile phase (methanol 100%) was delivered by an HPLC pump (model 9012; Varian, Mississauga, Ont., Canada) at a flow rate of 3 mL/min. In total, 150 mL fractions were collected using an automated collector (Dynamax FC-4; Varian). Fractions were analyzed and pooled based on the similarity of their HPLC profiles (Fig. 1) and tested for the stimulation of glucose uptake in an *in vitro* bioassay as described in Section 2.3 at a concentration of 200 μg/mL.

The isolation and purification of compounds from sub-fractions of ethyl acetate soluble fraction of *V. vitis* was achieved on a 1200 series preparative HPLC system (Agilent Technologies, Santa Clara, CA, USA), equipped with an autosampler with a 2 mL loop, a binary pump (flow rate range 5-100 mL/min), DAD and a fraction collector. A Gemini C18 reversed phase column (4.6×250 mm, particle size 10 μm) (Phenomenex, Torrance, CA, USA) was used to monitor the fractionation process and for scaling up for the isolation of the compounds from target fractions on a preparative scale Gemini C18 reversed phase column (21.2×250 mm, particle size 10 μm) (Phenomenex). Preparative scale isolation of the most active fractions was achieved by using a binary solvent system of solvent A (0.05% aqueous trifluoroacetic acid) and solvent B (100% ACN). The gradient elution program afforded a total of nine compounds from the two fractions.

LC-MS analysis of the crude *V. vitis* berry extract, its fractions, and the isolated compounds was performed on an HPLC-DAD-atmospheric pressure chemical ionization (APCI)-MSD system (Agilent Technologies, model 1100) which consisted of an autosampler with a 100 μL loop, a quaternary pump (maximum pressure, 400 bar), a column thermostat, a DAD and APCI-MS. The separations were achieved on an YMC-

ODS-AM, 100 mm×4.6 mm id, particle size 5 µm (YMC, Kyoto, Japan). The mobile-phase system consisted of water (solvent A) and ACN (solvent B). The optimized elution conditions were a linear gradient of 5-100% B in 35 min, the column was washed for 5 min at 100% B, brought back to starting mobile-phase composition in 0.1 min and equilibrated for 7 min before next injection. The HPLC separations were monitored at 290, 325, and 520 nm.

Mass spectrometric characterization was performed in both positive and negative ionization modes. For positive ionization mode, the optimized spray chamber conditions were: drying gas flow rate of 6 L/min, nebulizer pressure of 40 psig, drying gas temperature of 300°C, vaporizer temperature of 400°C, capillary voltage of 3000 V, and corona current of 3 µA. For negative ionization mode, the conditions were: drying gas flow rate of 6 L/min, nebulizer pressure of 60 psig, drying gas temperature of 350°C, vaporizer temperature of 400°C, capillary voltage of -3000 V, and corona current of 15 µA. APCI was conducted at 300°C with the vaporizer at 400°C; nebulizer pressure, 40 psig; nitrogen (drying gas) flow rate, 6 L/min; fragmentation voltage, 20 V; capillary voltage, 3000 V; corona current, 3 µA. The MS was operated in scan mode within 100-800 amu with fragmentation voltages of 20 and -160 V for positive and negative ionization, respectively.

The identification of the isolates was achieved by: (i) the comparison of UV absorption spectra against those from a custom metabolomics library consisting of 140 pure reference phenolic compounds [20]; (ii) co-chromatography with reference standards; (iii) the confirmation of the presence of characteristic ions; (iv) the comparison of the recorded 1H and 13C-NMR spectra (Avance 400 MHz NMR spectrometer; Bruker BioSpin, Billerica, MA, USA) with published spectra. Isolates were quantified by generating five-point linear calibration curves on the basis of area under the peaks recorded at: 325 nm, reference off, bandwidth 4 for phenolics; 290 nm, reference off, bandwidth 4 for procyanidins and catechins; 520 nm, reference off, bandwidth 4, for anthocyanins.

Results

V. vitis berry extract stimulates glucose uptake in C2C12 myotubes

An 18 h treatment with 200 µg/mL of the crude ethanol extract of *V. vitis* berries stimulated glucose uptake in C2C12 skeletal muscle cells by 31±7%, *n*=6 (Fig. 1). These results are comparable to those reported in the previous screening study in which an earlier collection of the same species was tested [13]. This stimulation of muscle cell glucose uptake was quantitatively similar to that obtained after 15 min treatment with 100 nM insulin (positive control; data not shown).

Bioassay guided fractionation, isolation, and identification of active principles

In order to identify its active principles, the *V. vitis* berry extract was fractionated using a multi-step approach guided by the enhancement of glucose uptake activity in C2C12 cells treated 18 h. The fractionation scheme and activity results obtained at every step are shown in Fig. 1. All fractions were tested at 200 µg/mL and all isolates at 100 µM. The crude ethanol extract was first fractionated into ethyl acetate-soluble and -insoluble fractions. Only the ethyl acetate-soluble fraction showed a significant stimulation of glucose uptake (41±5% above DMSO; *n*=3) and was selected for further fractionation on a Sephadex LH20 column.

This yielded six subfractions pooled according to similar HPLC profiles (see Section 2). Of these, subfractions 2 and 3 showed significantly higher stimulation of glucose uptake than the original *V. vitis* berry extract and other fractions (37±3% and 52±4%, respectively; *n*=6). These two fractions were selected for further fractionation.

Using preparative HPLC chromatographic fractionation, five compounds were isolated from subfraction 2 (Figs. 2 and 3; Table 1): *p*-coumaroyl-D-glucose; *p*-hydroxybenzoic acid; *p*-coumaric acid; benzoic acid; quercetin. Of these, only quercetin stimulated uptake when tested at 100 µM (37±9%; *n*=6).

Finally, seven compounds were identified from subfraction 3: quercetin-3-*O*-galactoside; quercetin-3-*O*-glucoside; an unidentified quercetin-3-*O*-glycoside; catechin; epicatechin; cyanidin-glucoside; cyanidin-galactoside (Figs. 2 and 3, Table 1). The first five of these compounds were isolated and the three quercetin-3-*O*-glycosides were found to induce a significant enhancement of glucose uptake at 100 µM (59±2%, 38±4%, and 24±3%, respectively; n=6). Cyanidin glycosides were also tested and found to be inactive (results not shown).

The identity of the purified compounds was confirmed by a combination of LC-MS and NMR and by comparison of their physicochemical properties with those reported in the literature [30] or with those of reference compounds. The predominant phenolic acid present in the berries was *p*-coumaric acid (33.8±0.6 µg/g dry weight). The predominant flavonols present in the berries were quercetin-3-*O*-glucoside (19.2±1.2 µg/g dry weight) and quercetin-3-*O*-galactoside (15.9±0.4 µg/g dry weight) (Table 1).

V. vitis crude extract and its active principles increase activity of the AMPK signaling pathway not of the insulin receptor pathway in C2C12 myotubes

To understand the mechanism mediating the effect of *V. vitis* berry extract on skeletal muscle cell glucose uptake, we evaluated the activity of the two main signaling pathways that regulate rate of glucose uptake in this cell-type: the insulin-receptor pathway and the AMPK pathway. Following an 18 h treatment in C2C12 cells, there was no indication of increased phosphorylation of Akt (Fig. 4A), a marker of the former pathway. Stimulation with 100 nM insulin for 30 min produced a clear activation of this enzyme. In contrast, treatment with the extract increased the phosphorylation of the AMPK effector ACC (Fig. 4B). AICAR, an AMP mimetic and known activator of AMPK signaling, served as a positive control and also greatly enhanced phosphorylation of ACC. Concordant with the activity of the crude extract, treatment of C2C12 cells for 18 h with 50 or 100 µM quercetin, quercetin-3-*O*-galactoside or quercetin-3-*O*-glucoside did not increase phosphorylation of Akt (Fig. 4A) but increased phosphorylation of ACC (Fig. 4B). Total content of ACC was not significantly altered by any treatment.

V. Vitis berry extract and quercetin, but not quercetin glycosides, inhibit respiration in isolated mitochondria

AMPK is highly sensitive to metabolic stress such as that occurs when energy transduction is disrupted. To test whether *V. vitis* berry extract and its active principles may have caused such a disruption, we assessed the effect of these products on respiration of isolated mitochondria. Succinate-supported rates of basal and ADP-stimulated oxygen consumption were measured in rat liver mitochondria treated with vehicle, 200 µg/mL crude extract, or 25-100 µM of quercetin or quercetin glycosides. The crude extract had no stimulatory effect on the rate of basal O_2 consumption but induced a mild inhibitory effect on the rate of ADP-stimulated O_2 consumption (Fig. 5), reducing the capacity for ATP synthesis by 9±3%. This pattern of disruption of mitochondrial function is consistent with an inhibition of ATP synthase. The quercetin aglycone produced a similar, but more pronounced inhibitory effect: at 25 and 100 µM, capacity was inhibited by 40±10% and 85±5%, respectively (Fig. 6A). The quercetin glycosides had much less effect than the aglycone, only decreasing capacity by 3-7% at 100 µM (Fig. 6B).

Quercetin does not increase the rate of secretion of acid equivalents or reduce intracellular ATP

Since quercetin powerfully inhibited respiration in isolated mitochondria, it was hypothesized that it would induce a compensatory increase in flux through anaerobic glycolysis and therefore an increase in the rate of secretion of acid equivalents. To test this, the pH of the culture medium of H4IIE hepatocytes and C2C12 muscle cells was spectrophotometrically assessed at several time points over a 4 h treatment with quercetin. Quercetin at either 25 or 100 µM did not significantly influence the rate of acidification of the medium of C2C12 or H4IIE cells (Fig. 7). In contrast, the positive control uncoupling compound FCCP greatly enhanced this rate.

Similarly, it was pertinent to verify if the metabolic stress induced by quercetin would negatively impact the intracellular ATP concentration. Again, neither 25 nor 100 µM of quercetin induced a drop in ATP in H4IIE hepatocytes over a 6-h period (Fig. 8); instead, cellular ATP was paradoxically increased after 3 h of treatment. FCCP used as a positive control transiently decreased content of ATP in H4IIE hepatocytes after 1 h of treatment.

Discussion

Aboriginal populations worldwide are susceptible to metabolic disorders related to lifestyle changes. Indeed, the incidence of obesity and diabetes in these populations is the highest in the world [1][7][31]. When this predisposition is coupled with a cultural disconnection with modern pharmaceuticals, the rate of diabetic complications and the associated social costs can become staggering. In an effort to remedy the situation in Canadian aboriginal populations facing these problems, our team has been working towards identifying safe and efficacious alternative treatment options for diabetes based on these populations' own traditional medicine and associated pharmacopea. In collaboration with the Cree of Eeyou Istchee (James Bay area of Que., Canada), we have used a novel ethnobotanical approach [10] to identify relevant medicinal plant species used to treat symptoms of diabetes. Follow-up studies screening the antidiabetic activity of extracts of these species have revealed eight products capable of enhancing glucose uptake in skeletal muscle cells [12][13].

The effects of all seven products to emerge from the first screening project [12] were found to be mediated by AMPK as a response to metabolic stress resulting from a disruption of mitochondrial energy transduction [14], a mechanism similar to that of the biguanide oral anti-hyperglycemic drug Metformin [17]. The purpose of this study was to evaluate whether the effects of a new product identified in our second screening study [13], the extract of the berries of *V. vitis idaea*, are also mediated by such a mechanism and to simultaneously isolate and identify the compounds responsible for this activity using our phytochemical expertise with *Ericacea* species [20][21]. The identification of active compounds will be useful for standardizing the activity of different preparations of the plant product and may also provide insight into the activity of other small berries used for the treatment of diabetes in various parts of the world.

AMPK is recognized as an important therapeutic target for diabetes [15][16]. Indeed, the effects of Metformin are mediated through this metabolic master enzyme and transducer of metabolic stress. Upon activation by an increase in the cellular ratio of AMP to ATP, AMPK serves to restore energy homeostasis by increasing flux through energy-

producing pathways and decreasing energy-consuming processes [32]. Energy production is increased by simultaneous enhancement of uptake and oxidation of lipids and carbohydrates. Other tissue-specific effects include the insulin-like inhibition of hepatic glucose output and the translocation of Glut-4 glucose transporters in skeletal muscle, activities that contribute to a systemic anti-hyperglycemic effect [17][33-35]. In addition to acute actions for restoring energy homeostasis, the activation of AMPK produces long-term adaptive effects, such as increased capacity for substrate uptake and oxidation, that confer protection against future metabolic stresses [36-38].

Many plant products are known to activate AMPK [39-48], including compounds isolated from *Galega officinalis* and from which Metformin is derived [49][50]. AMPK is not activated directly by these products, but rather as a consequence of the metabolic stress that they induce [39]. These compounds tend to be plant defensive metabolites that protect against microorganisms by disrupting well-conserved energy transduction pathways such as mitochondrial oxidative phosphorylation [18]. Several compounds of the flavonoid family are known to dissipate the mitochondrial proton gradient (*i.e.* uncoupling), while compounds such as metformin and oligomycin have been shown to inhibit electron transport or ATPsynthase respectively [18][42][51][52]. Our recent study of the mechanism of action of medicinal plant products, in which we demonstrated that the extracts of seven species all acted through AMPK, revealed both uncoupling- and inhibitory-type activities [14]; interestingly, in most cases both types of disruption were observed concurrently, perhaps suggestive of a combination of active principles. In this study, the extract of *V. vitis* berries also induced an activation of AMPK that can be explained by a disruption of mitochondrial function. This disruption was observed to be purely of the inhibitory type, resulting in a mild decrease in the rate of ADP-stimulated oxygen consumption in isolated mitochondria, with no effect on the rate of basal consumption. As with species tested in our previous study, *V. vitis* berry extract did not stimulate the insulin-signaling pathway. These results reinforce the notion that disruption of energy transduction and subsequent activation of AMPK is a simple mechanism that may explain the activity of several antidiabetic plant products used by cultures throughout the world. It is a mechanism that likely requires less molecular specificity than the

activation of the insulin receptor-signaling pathway. This pathway was found not to be stimulated by the extract of *V. vitis* berries, or by the plant products tested in our previous study.

Fractionation of *V. vitis* berry extract guided by muscle cell glucose uptake resulted in the isolation of quercetin-3-*O*-glycosides as main active principles. At 50 μM, these compounds enhanced basal glucose uptake by up to 59% following an 18 h treatment, an effect significantly greater than that of 100 nM insulin. These compounds were observed to increase the phosphorylation of ACC, thereby confirming that their mechanism of action was the same as that of the crude extract. However, unlike the crude extract, the quercetin glycosides failed to inhibit mitochondrial respiration. In contrast, the aglycone of these compounds, a minor component of a less active fraction, was found to both stimulate the AMPK pathway and to potently inhibit the rate of ADP-stimulated oxygen consumption. Such an inhibitory effect of quercetin on ATP synthase has been reported by others and has recently been attributed to direct binding of quercetin to the F1-ATPase [53]. The sugar moiety of the glycosides reduces the lipophilicity of quercetin and may therefore prevent the compound from permeating the mitochondrial inner membrane. Indeed, it is widely accepted that flavonoids are often glycosylated in plants as a mechanism for facilitating their handling or sequestration. It is also possible that the hydroxyl group at position 3, replaced by the sugar moiety, is essential for the activity of quercetin. As the amount of quercetin aglycone contained in the extract is insufficient to account for the inhibition of mitochondrial respiration and the activation of AMPK, these findings suggest that quercetin-glycosides may be hydrolyzed to the aglycone form in order to become active. A less likely alternative is that quercetin glycosides may activate AMPK directly without inducing metabolic stress. In any case, the combined action of quercetin and its 3-*O*-glycosides appear to underlie the majority of the action of *V. vitis* on muscle cell glucose transport.

Quercetin does not appear to produce the dangerous side effects that can occur with powerful disruptors of oxidative phosphorylation. First, quercetin did not increase the rate of extracellular acidification, a marker of the contribution of anaerobic glycolysis to ATP

synthesis. Second, quercetin did not decrease cytosolic ATP concentration following 1 or 3 h of treatment. Both observations support the notion that the metabolic stress induced is of low magnitude and short-lived, not affecting ATP concentration nor requiring a significant upregulation of glycolysis. Interestingly, ATP concentration was actually increased above normal by treatment with quercetin. This may be explained if the AMPK-derived signal for increased ATP synthesis through lipid and carbohydrate oxidation is longer-lived than the metabolic stress itself, resulting in an overshoot of ATP content. This ATP surfeit may also account for the observed tendency towards a paradoxically reduced rate of flux through anaerobic glycolysis. Together, these results can also be taken to indicate that quercetin is an easily metabolized compound, a conclusion supported by pharmacokinetic studies [22].

Concluding remarks

In summary, the results presented here demonstrate that quercetin and quercetin glycosides are active principles responsible for the enhancement of muscle cell glucose uptake by the extract of *V. vitis* berries. Quercetin and quercetin glycosides exert antidiabetic activity through the AMPK signaling pathway, activated as a response to the action of the quercetin aglycone on mitochondrial energy transduction. This mechanism concords with the anti-hyperglycemic activity of quercetin reported by others [54][55]. Quercetin and quercetin glycosides are found in the berries of other members of the *Vaccinium* family used against diabetes and are likely to be active principles in these species as well. Preparation of *V. vitis* berries hold good potential for the treatment of diabetes in Canadian aboriginal populations.

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Figure Legends

Figure 1. Phytochemical fractionation of *V. vitis* berry extract guided by muscle cell glucose-uptake activity. Values in brackets represent activity expressed as percentage change in the rate of basal glucose uptake relative to the vehicle control (0.1% DMSO) following an 18 h treatment with respective fractions at 200 µg/mL or isolates (Fig. 2 and Table 1) at 100 µM.

Figure 2. HPLC chromatograms of *V. vitis* berry crude extract (A), subfractions 2 (B) and 3 (C) of its ethyl acetate-soluble fraction. Absorbance at 325 and 520 nm (milliabsorbance units) is plotted against retention time (in min). Twelve constituent compounds were identified using a metabolomics approach, as described in Section 2.1. The identity of these peaks is listed in Table1.

Figure 3. Chemical structures of the 12 isolated constituents *V. vitis* berry ethanol extract.

Figure 4. *V. vitis* berry extract and its active principles stimulate the AMPK signaling pathway but not the insulin receptor pathway. C2C12 skeletal muscle cells were treated for 18 h with either 0.1% DMSO (vehicle), 200 µg/mL of *V. vitis* berry extract, or 50 and 100 µM of quercetin, quercetin-3-*O*-glucoside or quercetin-3-*O*-galactoside. Phosphorylation of the insulin receptor pathway marker Akt (A) and of the AMPK effector ACC was measured by western immunoblot. Insulin (100 nM) and AICAR (2 mM) applied for 30 min served as positive controls.

Figure 5. The *V. vitis* berry extract induces a mild instantaneous inhibition of respiration in isolated rat liver mitochondria, as illustrated by a representative oxygen consumption tracing. Mitochondria (1 mg mitochondrial protein) were treated with vehicle (0.1% DMSO) or 200 µg/mL of extract and the rates of succinate-supported basal and ADP-

stimulated oxygen consumption were measured. As compared to control, extract-treated mitochondria exhibited an unchanged rate of basal oxygen consumption but a mildly inhibited rate of ADP-stimulated oxygen consumption. Values represent rate of consumption in nmol O/min/mg protein. Experiments were repeated in three different mitochondrial preparations.

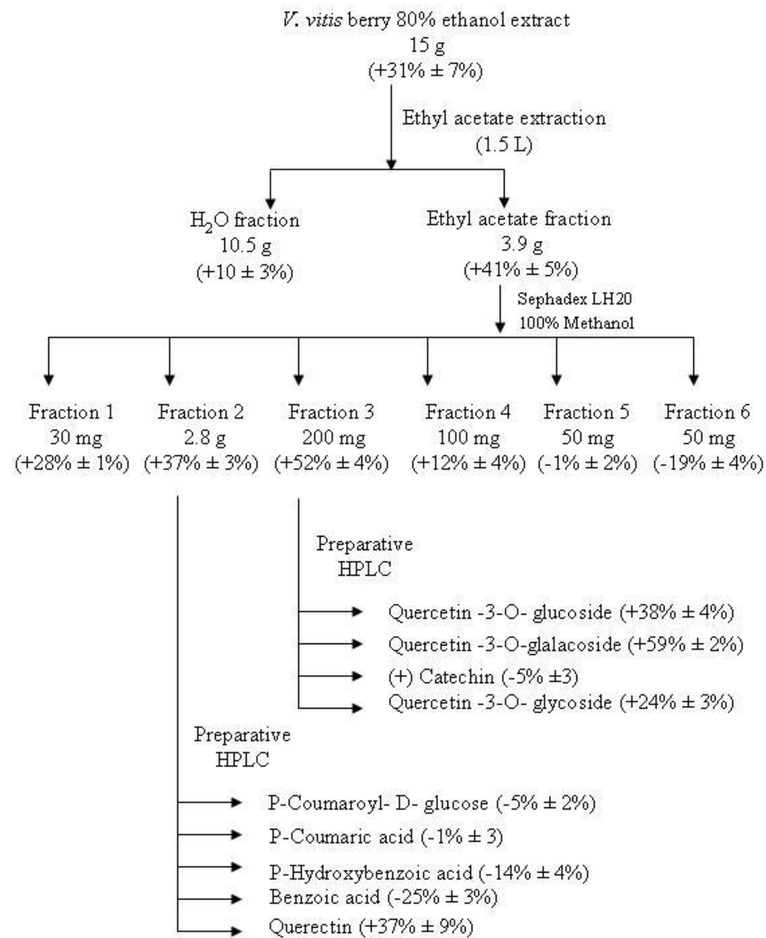
Figure 6. Quercetin (A), but not quercetin-3-*O*-glycosides (B), induces an important instantaneous and dose-dependent inhibition of respiration in rat liver mitochondria, as illustrated by representative oxygen consumption tracings. Values represent rate of consumption in nmol O/min/mg protein. Experiments were repeated in three different mitochondrial preparations.

Figure 7. Quercetin does not increase the rate of secretion of acid equivalents by C2C12 (A) or H4IIE (B) cells. Acidification of the cell medium. The pH of the culture medium was assessed with a Phenol Red-based spectrophotometric assay at several time points. Change in pH was expressed as the cumulative secretion of acid equivalents. FCCP (5 μM) was used as a positive control. Quercetin treatment was not significantly different from vehicle (0.08% ethanol) in either cell line and at either concentration (25 and 100 μM). Data are mean±SEM for two experiments of four to five replicates *per* condition *per* time point.

Figure 8. Quercetin does not reduce intracellular ATP concentration in H4IIE hepatocytes. Cytosolic ATP content was measured in H4IIE hepatocytes using a luminescent ATP assay. FCCP (5 μM) was used a positive control. Quercetin treatment was not significantly different from vehicle (0.1% DMSO) at either concentration (25 and 100 μM). Data are expressed as mean±SEM of two experiments of four to five replicates *per* condition *per* time point.

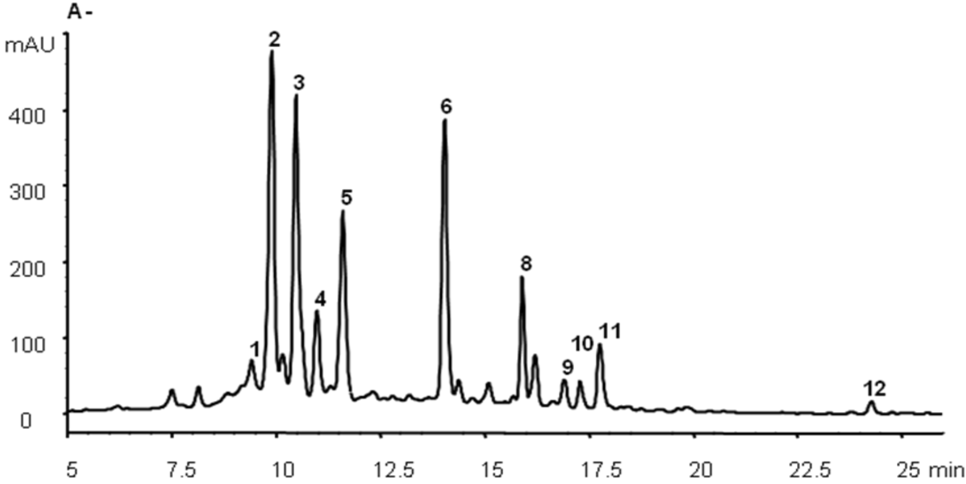
Figures

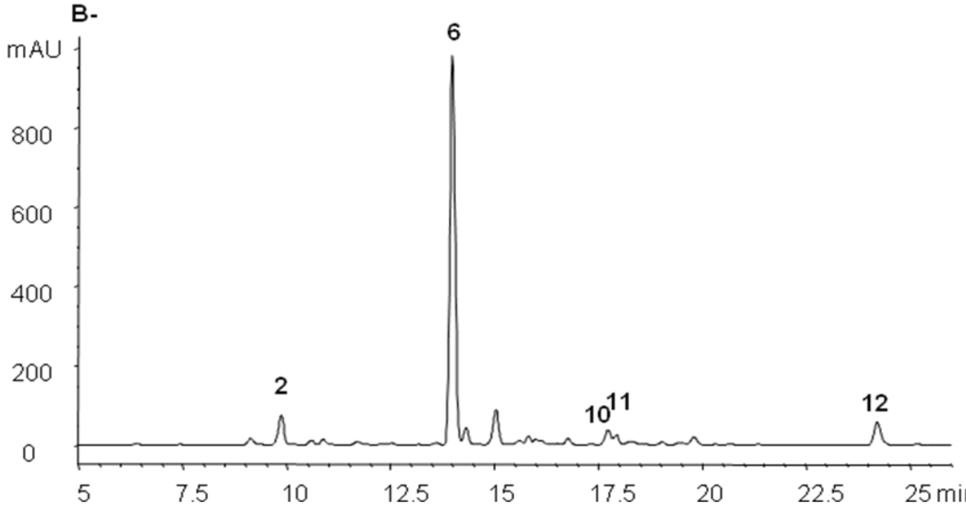
Figure 1



Values in brackets represent activity expressed as percentage change in the rate of basal glucose uptake relative to the vehicle control (0.1% DMSO).

Figure 2





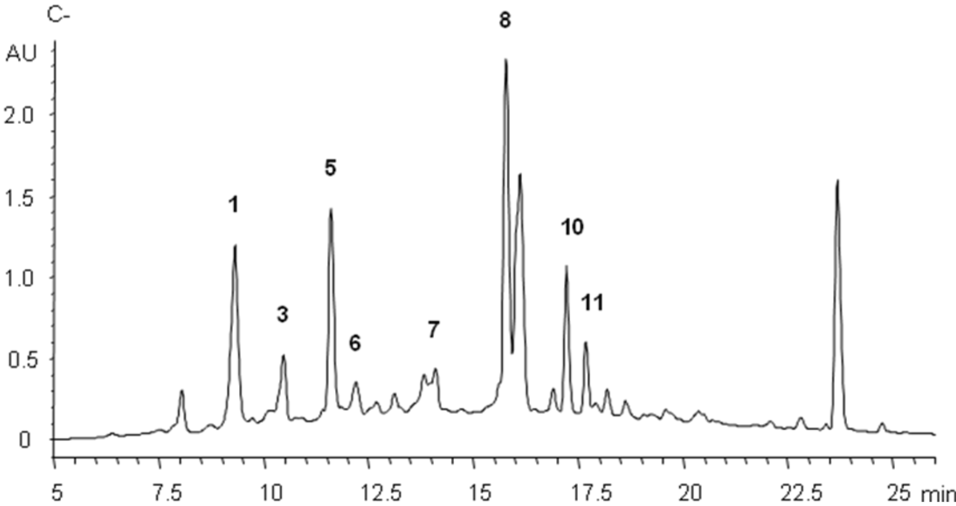


Figure 3

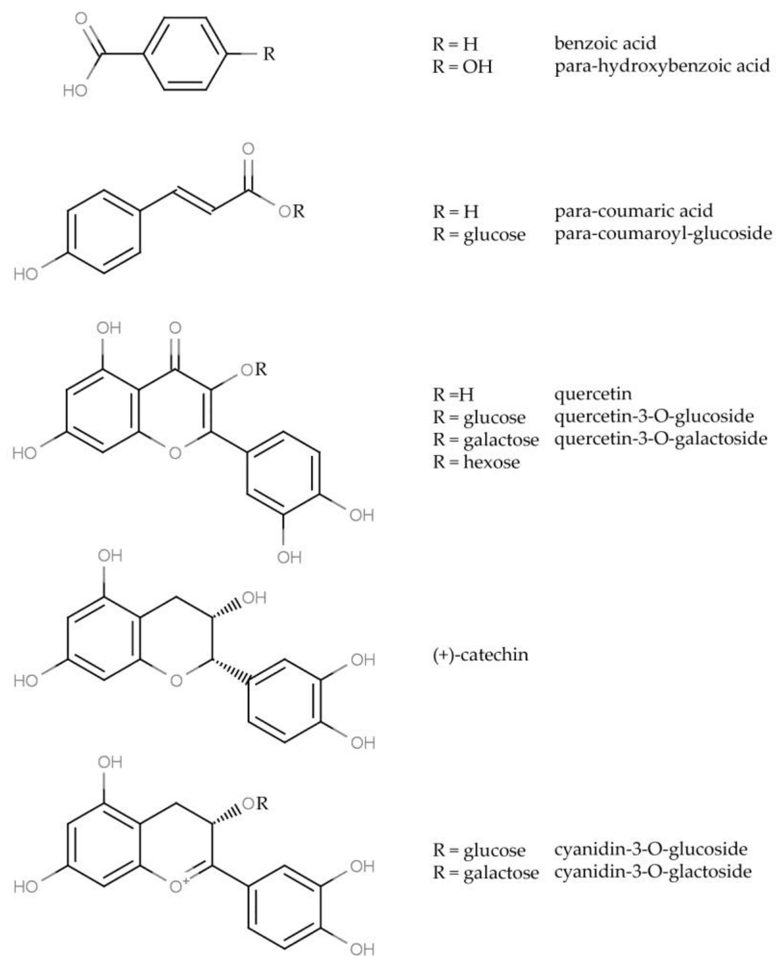


Figure 4

Figure 4A

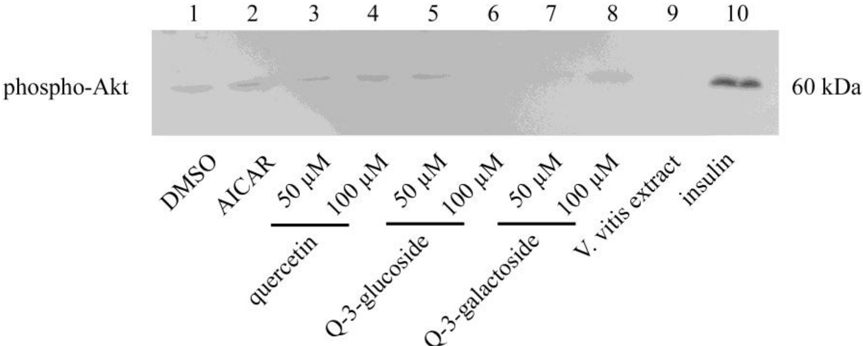


Figure 4B

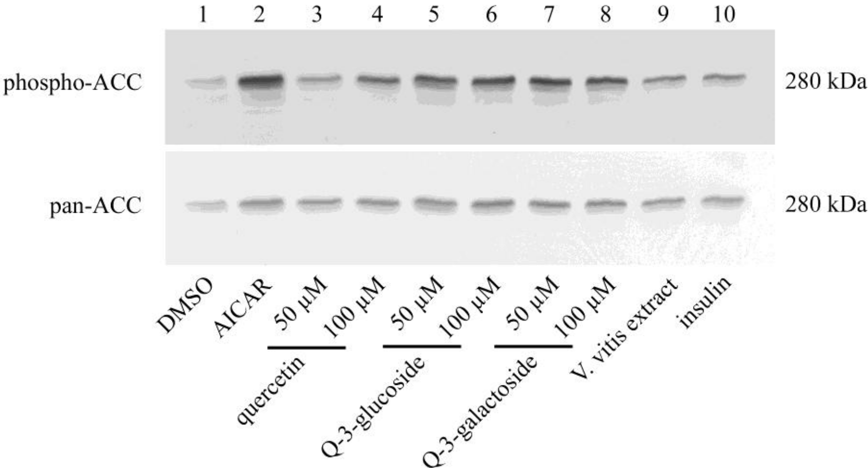


Figure 5

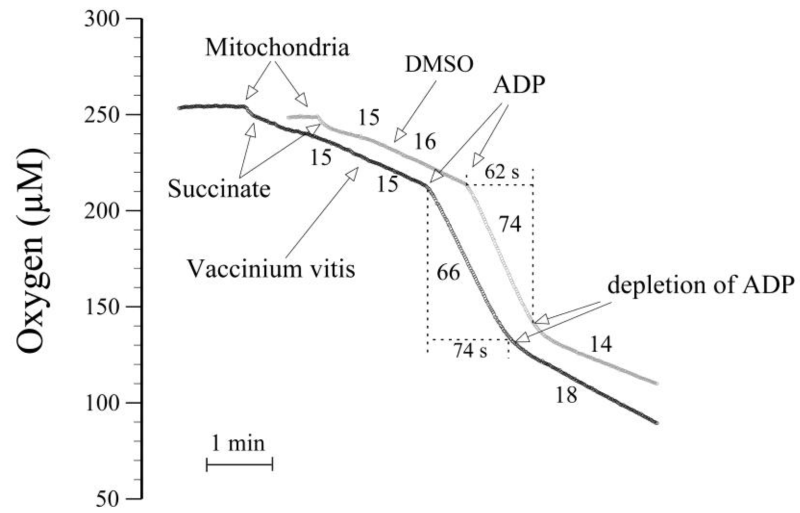


Figure 6

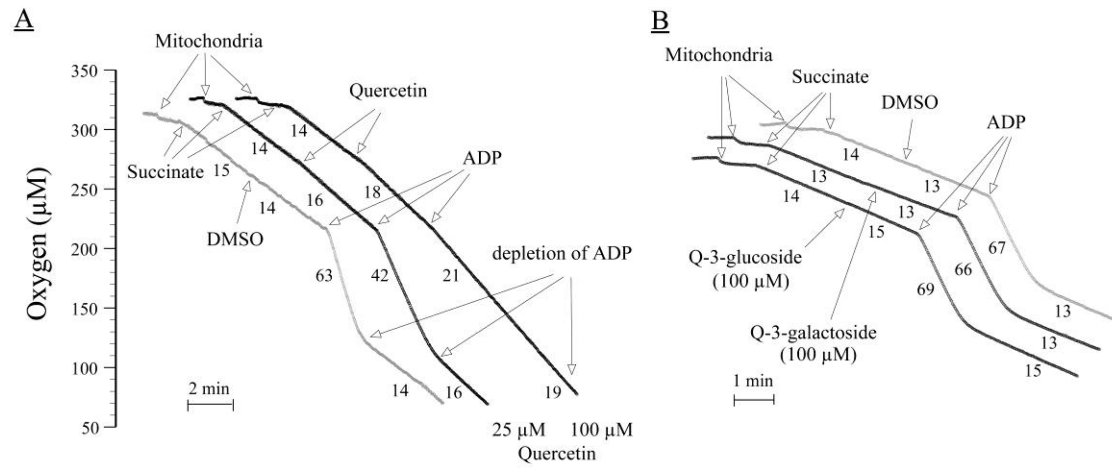


Figure 7

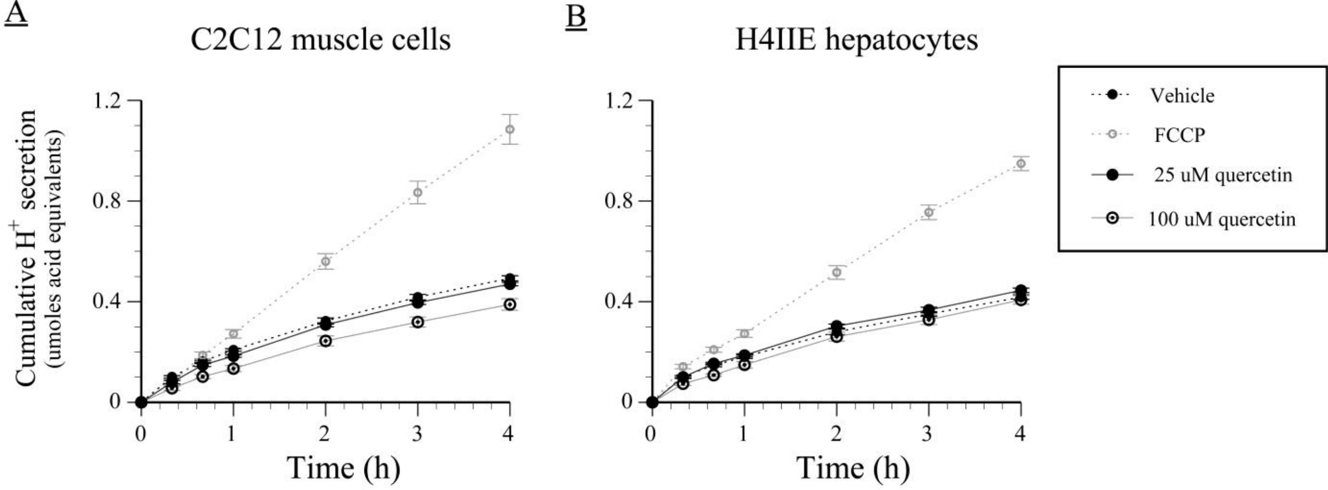


Figure 8

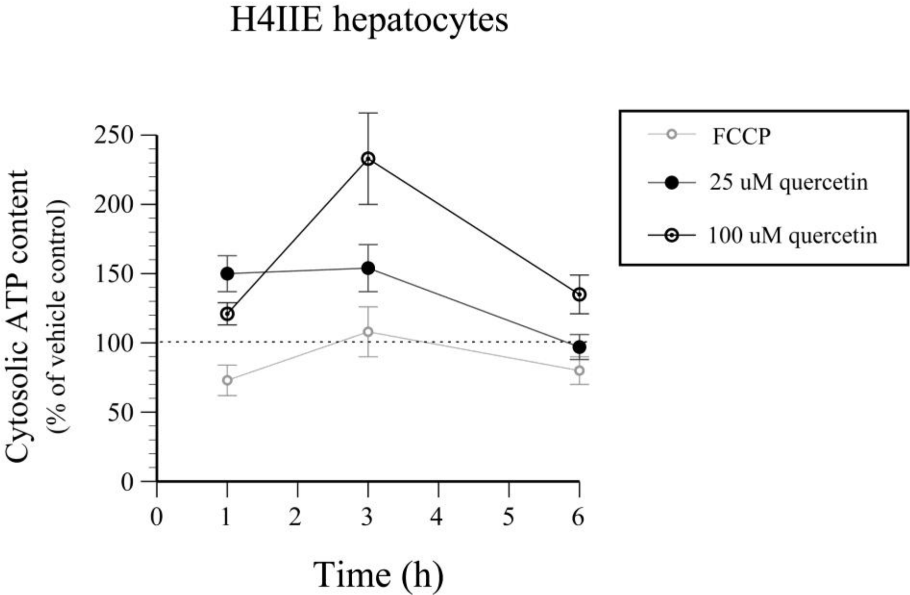


Table 1 Yield of *V. vitis* berry extract constituents

	Compound	Content (μg /g dry weight of berries)
1	(+)-catechin	2.8 ± 0.7
2	*p*-coumaric acid	33.8 ± 0.6
3	cyanadin-glucoside	30.4 ± 0.7
4	cyanadin galactoside	34.4 ± 0.3
5	*p*-coumaroyl-D-glucose	22.5 ± 0.4
6	epicatechin	3.8 ± 1.4
7	*p*-hydroxybenzoic acid	3.6 ± 0.2
8	benzoic acid	35.1 ± 0.2
9	quercetin-3-*O*-galactoside	15.9 ± 0.4
10	quercetin-3-*O*-glucoside	19.2 ± 1.2
11	unknown quercetin-3-*O*-glycoside	21.9 ± 1.3
12	quercetin	2.3 ± 0.3

3. Article 2

Structural constraints and the importance of lipophilicity for the uncoupling activity of naturally-occurring caffeic acid esters with potential for the treatment of insulin resistance

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Authors' contribution

I performed the experimental work, data analysis and wrote the paper.

Louis Martineau contributed to the elaboration of protocols, interpretation of data and correction of the paper.

Muhammad Asim synthesized some of the caffeic acid derivatives in the laboratory of Dr Tony Durst.

Diane Vallerand performed the mitochondrial respiration assay.

Dr Pierre Haddad is my supervisor.

Short Title: Mitochondrial uncoupling by caffeic acid derivatives

Keywords: Naturally occurring phenolic compounds; Mitochondrial energy transduction; Uncoupling of oxidative phosphorylation; Adenosine monophosphate (AMP)-activated protein kinase signaling pathway; Glucose uptake; Insulin resistance

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Abstract

Caffeic acid phenethyl ester (CAPE) has recently been shown to potently stimulate glucose uptake in cultured skeletal muscle cells through the AMPK pathway and therefore to have anti-diabetic potential. We report here that CAPE increases glucose uptake in C2C12 muscle cells by 225 ± 21% at 50 μM, and that activation of AMPK is a consequence of the metabolic stress resulting from an uncoupling-type disruption of mitochondrial function (complete uncoupling at 50 μM). We also observe that the therapeutic potential of CAPE is offset by its high potential for toxicity. The purpose of this study was therefore to identify other active caffeic acid derivatives, evaluate their ratio of activity to toxicity, and elucidate their structure–activity relationship. Twenty naturally occurring derivatives were tested for glucose-uptake stimulating activity in C2C12 cells following 18 h of treatment and for uncoupling activity in isolated rat liver mitochondria. Cytotoxicity was assessed in C2C12 cells by the release of lactate dehydrogenase over 18 h. In addition to CAPE, four compounds were identified to be active, both stimulating glucose uptake and uncoupling isolated mitochondria. Activity required that the caffeic acid moiety be intact and that the compound not contain a strongly ionized group. Both activity and toxicity were found to be well-correlated to predicted lipophilicity. However, two compounds exhibited little to no toxicity while still stimulating glucose uptake by 65–72%. These results support a therapeutic potential for this family of compounds and provide the framework for the design of alternatives to Metformin with an optimized balance of safety and activity.

Introduction

Insulin resistance is a major health concern throughout the world. It is a precursor to the diseases that make up the metabolic syndrome, including type II diabetes, cardiovascular disease, and nonalcoholic fatty liver disease, and is linked to excess adiposity, sedentary lifestyle, poor dietary habits, and aging [1]. At the cellular level, the development of insulin resistance is caused in part by abnormal accumulation and metabolism of lipids, as well as by mitochondrial dysfunction [2] and [3].

A key therapeutic target of pharmacological interventions for improving insulin sensitivity is the master metabolic regulatory enzyme AMP-activated protein kinase (AMPK) [4] and [5]. This enzyme is an extremely sensitive monitor of energy homeostasis, specifically of the concentrations of ATP and AMP. Upon activation under conditions of metabolic stress, AMPK triggers cytoprotective programs for acutely upregulating ATP production and downregulating non-essential energy expenditure, as well as transcriptional events that confer enhanced protection against future metabolic stress [6] and [7]. In the context of insulin resistance and compromised glycemic control, the activation of AMPK produces insulin-like effects that contribute to the normalization of hyperglycemia, namely the inhibition of glucose output by liver cells and the stimulation of glucose uptake by skeletal muscle cells. Furthermore, acute stimulation of fat oxidation and increased mitochondrial density are also AMPK-mediated effects that are relevant to the protection and restoration of insulin sensitivity in liver and muscle.

AMPK mediates the actions of the successful insulin-sensitizer Metformin and of the other members of the biguanide family [8]. These compounds indirectly activate AMPK by inducing a partial and transient inhibition of mitochondrial energy transduction and thereby disrupting energy homeostasis. The biguanides are effective at inhibiting hepatic glucose production [9] and [10]. However, their effect on skeletal muscle, the major site of glucose disposal, is more limited [11]. Also, Metformin and the other biguanides are associated with a potential for toxicity in the form of lactic acidosis [12] since compromised aerobic metabolism must be compensated by upregulation of anaerobic glycolysis. It appears that this potential is proportional to activity and that safety can only

be improved at the cost of efficacy. Indeed, the more powerful biguanides have been removed from many markets, leaving only Metformin [9] and [10], a product requiring doses of multiple grams per day.

There is an impetus to identify novel activators of AMPK that are at least as safe as Metformin but that are more potent and more efficacious at stimulating glucose uptake and inducing other therapeutically relevant AMPK-mediated effects in skeletal muscle. A starting point may be other types of compounds that also disrupt mitochondrial function but which do so through different mechanisms. Inhibitors of ATP synthase [13], [14] and [15] or dissipators of the mitochondrial proton gradient that drives conversion of ADP to ATP (i.e. uncouplers) are found in nature where they are used by many plant species to defend against predatory microorganisms [13] and [16]. While lactic acidosis will always remain a potential complication of any disruptor of aerobic metabolism, it may nevertheless be possible to identify in nature classes of compounds with a more favorable toxicity-to-activity relationship, especially if focus is placed on small phenolic compounds presumably easily metabolized by higher organisms. Indeed, activity may be uncoupled from this type of toxicity if mitochondrial effects are short-lived and of no more than sufficient duration to promote the activation of AMPK. Of interest is the naturally occurring small phenolic caffeic acid phenethyl ester (CAPE). This compound has recently been observed to activate AMPK kinase and to robustly and potently stimulate glucose uptake in skeletal muscle cells [17]. The purpose of the present study was to assess whether this effect of CAPE is a consequence of disruption of mitochondrial function and to test related compounds for similar activity. The results indicate that CAPE is an uncoupler of oxidative phosphorylation, that other closely related derivatives also exhibit both uncoupling activity and glucose-uptake stimulating activity, and that some of these compounds such as caffeic acid ethyl ester (CAEE) and caffeic acid methyl ester (CAME) exhibit useful activity with little to no associated cytotoxicity.

Materials and Methods

Source of compounds and reagents

CAPE and other caffeic acid derivatives (summarized in Table 1 and Fig. 4) were purchased from Sigma–Aldrich (Oakville, ON), with the exception of caffeic acid methyl ester, dihydrocaffeic acid, rosmarinic acid, ferulic acid methyl ester and ferulic acid ethyl ester purchased from Indofine Chemical Co. (Hillsborough, NJ), caffeic acid n-octyl ester and ferulic acid phenethyl ester purchased from LKT Laboratories Inc. (St.-Paul, MN), and dihydrocaffeic methyl ester, 4-hydroxycinnamic methyl ester, ferulic acid methyl ester, 2,4-dihydroxycinnamic acid methyl ester, and 4-(1-propenyl)-catechol synthesized as described in Sections Sections 2.2 and 2.3. Cell culture reagents were purchased from Invitrogen Life Technologies (Burlington, ON), unless otherwise noted. Other reagents were purchased from Sigma–Aldrich unless otherwise noted. Antibodies against phosphorylated (Ser 79) and pan-specific acetyl-CoA-carboxylase (ACC), phosphorylated (Ser 473) and pan-specific Akt, and β-actin were purchased from Cell Signaling Technology (Danvers, MA). Secondary HRP-conjugated antibodies were purchased from Jackson Immunoresearch (Cedarlane Laboratories, Hornby, ON).

Synthesis of methyl ester compounds

Solutions of dihydrocaffeic acid, 4-hydroxycinnamic acid, ferulic acid, and 2,4-dihydroxycinnamic acid (11.1 mM) in methanol (50 ml) were separately treated with a catalytic amount of concentrated H2SO4 and heated at reflux for 10 h. The reaction mixtures were cooled at room temperature and concentrated. The residues were dissolved in ethyl acetate and washed successively with water and brine. The ethyl acetate layers were dried over anhydrous MgSO4 and purified by column chromatography on silica gel to give dihydrocaffeic methyl ester (yellowish brown liquid), 4-hydroxycinnamic methyl ester (white powder), ferulic acid methyl ester (yellowish brown liquid), and 2,4-dihydroxycinnamic acid methyl ester (white powder). The identity and purity of these four compounds were confirmed by mass spectroscopy and by 1H and 13C NMR spectroscopy (Avance 400; Bruker BioSpin Corp., Billerica, MA).

Synthesis of 4-(1-propenyl)-catechol

4-(1-Propenyl)-catechol was synthesized according to a previously reported method [18]. Briefly, 3,4-dihydroxybenzaldehyde (7.24 mmol) and imidazole (2.3 equivalents) were dissolved in a 1:1 DMF/THF solution (10 ml). Teriary butyl dimethylsilyl chloride (2.2 equivalents) and 4-dimethylaminopyridine (trace) were added and the reaction mixture was stirred overnight at room temperature. The mixture was then diluted with distilled water (15 ml) and ether (25 ml) and then extracted with ethyl acetate (3 ml × 15 ml). The organic extracts were combined, dried over MgSO4, filtered, and evaporated under vacuum. The crude product was purified on a flash column. Elution with hexanes resulted in 1-(3,4-bis(tert-butyldimethylsilyloxy) benzaldehyde (compound 1) as a clear colorless oil. Ethylmagnesium bromide (3.0 M solution in THF; 1.5 equivalents) was added dropwise to a solution of compound 1 (1.09 mmol) in 10 ml of dry THF under nitrogen atmosphere at 0 °C. The resulting grayish solution was stirred for 30 min at 0 °C and left at room temperature for 1 h. The organic mixture was diluted with 10% HCl solution (2 ml) and extracted with ethylacetate (3 ml × 10 ml). The organic layers were combined, dried over MgSO4, filtered and concentrated under vacuum. The crude product was purified on a silica column. Elution with 15% ethylacetate in hexanes resulted in compound 2 as yellow oil. Compound 2 (0.75 mmol) was dissolved in methanol (5 ml) and few drops of concentrated HCl were added. The reaction mixture was refluxed for 2 h. The resulting yellowish mixture was diluted with distilled water and extracted with ethylacetate (3 ml × 10 ml). The organic layers were combined, dried over MgSO4, filtered and concentrated under vacuum. The crude product was purified on a silica gel column. Elution with 50% ethylacetate in hexanes resulted in 4-(1-propenyl)-catechol as yellow solid at a purity of greater than 95%. The identity and purity of this compound was confirmed by mass spectroscopy and by 1H and 13C NMR spectroscopy.

Estimation of pKa and log P

The acid-dissociation constant (pKa) for each ionizable group and the octanol–water partition coefficient (P), a predictor of lipophilicity, were estimated using the Marvin 5.1

Academic Package (ChemAxon Kft., Budapest, Hungary). Structures were inputted manually into MarvinSketch. The Marvin Protonation calculator plug-in was used to calculate pKa at a temperature of 37 °C. The Marvin Partitioning calculator plug-in was used to calculate log P of the neutral molecular species at an ionic strength of 0.1 M/dm3 Na+/K+ and 0.1 M/dm3 Cl−. Calculated log P and pKa were verified against published experimental values whenever these were available.

Cell culture

C2C12 murine skeletal myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA). C2C12 myoblasts were cultured in 6- or 12-well plates at 37 °C in a 5% CO2 environment in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Wisent, St-Bruno, QC) containing 10% fetal bovine serum and 10% horse serum (HS) and supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) as previously described [19], [20] and [21]. Upon reaching 80% confluence, serum content was reduced to 2% to induce differentiation into multinucleated myotubes over a period of 7 days. On the 6th day of differentiation, compounds solubilized in dimethyl sulfoxide (DMSO) and mixed in culture medium to achieve a final concentration of 50 μM in 0.1% DMSO were applied for 18 h prior to glucose uptake, western immunoblot, or LDH release assays.

3H-deoxyglucose uptake assay

Differentiated C2C12 myotubes grown in 12-well plates were treated for 18 h with 0.1% DMSO (vehicle control) or with 50 μM of CAPE or other caffeic acid derivative. The effects of CAPE resulting from a treatment of this duration have not previously been reported. The 50 μM concentration was selected for the testing of all compounds based on pilot studies indicating that 50 μM or below of a wide variety of naturally occurring small phenolics is typically well-tolerated by C2C12 myotubes over an 18 h period, with no effect on morphology and little to no effect on viability. Furthermore, a pilot dose–response study of CAPE indicated that glucose uptake following an 18 h treatment peaked at 50 μM

(not shown). Following treatment, cells were rinsed twice with Krebs-phosphate buffer (KPB; 20 mM HEPES, 4.05 mM Na2HPO4, 0.95 mM NaH2PO4, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 5 mM glucose, 0.5% BSA) at 37 °C and equilibrated in this buffer for 30 min. Some vehicle-control cells were treated during this time with 100 nM insulin to serve as a reference control. Following this, cells were washed twice in glucose-free KPB at 37 °C and then incubated for exactly 10 min in 0.5 µCi/ml of 2-deoxy-d-[1-3H]-glucose (TRK-383; Amersham Biosciences, Baie d'Urfé, QC) in this same buffer. Cells were then rapidly placed on ice and rinsed three times with ice-cold KPB, before lysis and scraping in 1 ml of 0.1 mM NaOH. Lysates were added to 4 ml of scintillation liquid cocktail (Ready-Gel 586601; Beckman Coulter Inc., Fullerton, CA) and radioactivity was measured in a scintillation counter (LKB Wallac RackBeta; Perkin Elmer, Montreal, QC).

Isolation of mitochondria from rat liver

Mitochondria were isolated from the liver of male Wistar rats (Charles River, St-Constant, QC) weighing between 225 and 250 g. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and underwent laparotomy. All experimental procedures were approved by the Université de Montréal Animal Experimentation Ethics Committee and animals were treated in accordance with guidelines of the Canadian Council on the Care and Protection of Animals. The portal vein was canulated while the hepatic artery and the infrahepatic inferior vena cava were ligated. The livers were flushed with 100 ml of Krebs–Henseleit buffer (25 mM NaHCO3, 1.2 mM, KH2PO4, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 2.1 mM CaCl2, 1.2 mM MgSO4) at ambient temperature and livers were removed and placed on ice. Mitochondria were isolated from 1 g of liver as described by Johnson and Lardy. Briefly, tissue was homogenized on ice using a Teflon potter homogenizer in ice-cold isolation buffer (10 mM Tris, pH 7.2, 250 mM sucrose, 1 mM EGTA). The homogenate was centrifuged at 600 × g for 10 min at 4 °C in order to remove cellular fragments. The supernatant was recovered and centrifuged at 12,000 × g for 6 min at 4 °C. The supernatant was discarded and the pellet was washed in ice-cold isolation buffer and recentrifuged. The pellet was then

washed in EGTA-free isolation buffer, and again recentrifuged. The final pellet containing viable mitochondria was resuspended in ice-cold EGTA-free isolation buffer and this preparation was kept on ice until respiration experiments. Protein content of the preparation was determined according to the Lowry method.

Mitochondrial respiration assay

O2 consumption was measured at 25 °C using a Clark-type oxygen microelectrode in a 1 ml volume temperature-controlled chamber with oxygen concentration sampled and recorded to a microcomputer at a frequency of 1 Hz (Oxygraph system; Hansatech Instruments, Norfolk, England) as previously described. Briefly, 1 mg of mitochondrial protein was added to 990 µl of respiration buffer (5 mM KH2PO4, pH 7.2, 250 mM ultra-pure sucrose, 5 mM MgCl2, 1 mM EGTA). Mitochondrial respiration was initiated by addition of the complex II substrate succinate (5 mM final concentration). After reaching a stable rate of basal O2 consumption (RBOC; State 4 respiration), vehicle alone or a caffeic acid derivative solubilized in DMSO was injected to achieve a final concentration of 50 µM of experimental compound in 0.1% DMSO. An increase in RBOC per mg mitochondrial protein was considered an uncoupling effect. DMSO used at 0.1% did not affect RBOC. Basal respiration was allowed to proceed for at least 30 additional seconds before the induction of oxidative phosphorylation (State 3 respiration) by the addition of 200 µM (final concentration) ADP. Each experimental session consisted of 10–12 experiments from a single mitochondrial preparation, including 3–4 vehicle control experiments to determine baseline values for the session. The effect of each experimental compound was evaluated as: (1) the increase in RBOC per mg protein (a measure of the magnitude of the uncoupling effect); (2) the decrease in functional capacity (FC) per mg protein (a measure of the magnitude of the uncoupling effect plus any additional inhibitory effect), where FC was defined as the difference of the rate of ADP-stimulated O2 consumption (RASOC) per mg protein (maximal functional rate of consumption) and RBOC per mg protein (rate of consumption driven by proton leak and not contributing to ATP synthesis). Calculations were as follows: the average FC per mg protein of the vehicle control experiments for a given session was calculated by subtracting the average RBOC per mg protein from the

average RASOC per mg protein. For (1) above, the absolute increase in RBOC per mg protein measured in a given experiment was expressed as a percentage of the average control FC per mg protein for the session. For (2) above, the FC per mg protein measured in a given experiment was expressed as a percentage of the average control FC per mg protein for the session to give the % residual FC. All compounds were tested in at least two different mitochondrial preparations.

Western immunoblot

Differentiated C2C12 myotubes grown in 6-well plates were treated for 18 h with 0.1% DMSO or with 50 µM of CAPE or of other caffeic acid derivative, and lysed for western immunoblot analysis. Some vehicle-control cells were treated with 2 mM 5-aminoimidazole-4-carboxamide-1-b-D-rubofuranozide (AICAR; Toronto Research Chemicals, North York, ON), a positive control for activation of the AMPK pathway, for 30 min immediately prior to lysis. Following treatment, plates were placed on ice and washed three times in ice-cold phosphate-buffered saline (PBS; 8.1 mM NaHPO4, 1.5 mM KH2PO4, pH 7.4, 137 mM NaCl and 2.7 mM KCl) and lysed in 250 µl of lysis buffer (25 mM Tris–HCl pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate 0.1% SDS) containing a commercial cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany) supplemented with 1 mM phenylmethanesulfonyl fluoride, as well as a cocktail of phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Cells were allowed to lyse for 15 min on ice, and then scraped into microcentrifuge tubes, periodically vortexed, and centrifuged at 600 × g for 10 min at 4 °C. Supernatants were decanted and stored at −80 °C until further analysis. Protein content was assayed by the bicinchoninic acid method (Thermo Scientific Pierce Protein Research, Rockford, IL) standardized to bovine serum albumin. Lysates were diluted to a concentration of 1.25 mg total protein per ml and boiled for 5 min in reducing sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). 20 µg of protein of each sample were separated on 10% polyacrylamide mini-gels and electrotransferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) overnight under 330 mA of

current at 4 °C. Membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline (20 mM Tris–HCl, pH 7.6 and 137 mM NaCl) containing 0.1% Tween 20 (TBST). Membranes were then incubated overnight at 4 °C in blocking buffer with primary antibodies at a concentration of 1:1000. Membranes were washed 5 times with TBST and incubated 1.5 h at ambient temperature in TBST with appropriate horseradish peroxidase-conjugated secondary antibodies at 1:50,000–100,000. Revelation was performed using the enhanced chemiluminescence method (Amersham Biosciences, Buckinghanshire, England) and blue-light-sensitive film (Amersham Biosciences). Experiments were repeated on 3 different passages of cells, each passage containing all conditions in parallel. All samples from a given passage were separated and transferred simultaneously to a single membrane. Quantification of the integrated density of bands was performed using a flatbed scanner (ScanJet 6100; Hewlett Packard, Palo Alto, CA) and NIH Image 1.63 software (National Institutes of Health, Bethesda, MD).

LDH release cytotoxicity assay

Differentiated C2C12 myotubes grown in 12-well plates were treated for 18 h with 0.1% DMSO or with 50 μM of CAPE or of other caffeic acid derivative. Medium was removed and kept on ice. Cells were rinsed in PBS and lysed in 1% Triton X-100. Lactate dehydrogenase (LDH) activity in medium and in lysates was assayed with the LDH-Cytotoxicity Assay Kit II (BioVision, Mountain View, CA). Medium LDH activity was expressed as a percentage of total (medium + lysate) LDH activity. Experiments were performed in triplicate.

Statistical analysis

All data are reported as the mean ± SEM of the indicated number of experiments. Results were analyzed by one-way analysis of variance using StatView software (SAS Institute Inc., Cary, NC). Statistical significance was set at $p \leq 0.05$. Non-linear regression analysis was performed by Prism 4.0 (GraphPad Software Inc., La Jolla, CA) using the

following sigmoidal dose–response equation: $y = bottom + (top - bottom)/(1 + 10((x50 - x)* Hill slope))$ where $x = \log P$ and $x50 = \log P$ resulting in half maximal effect.

Results

CAPE increases glucose uptake following an 18 h treatment

CAPE has recently been shown to induce an important AMPK-mediated stimulation of glucose uptake in skeletal muscle cells following a 1 h treatment [17]. A first objective of the present study consisted in testing the effect of CAPE on muscle cell glucose uptake following a longer treatment duration more conducive to the expression of AMPK-mediated transcriptional effects. Our previous studies on natural products that enhance basal glucose uptake through AMPK have shown that in C2C12 muscle cells an 18 h treatment invariably results in a more important effect than a 1 h treatment [19], [20] and [21], presumably due to transcriptional effects of AMPK [6], [7], [22] and [23]. Differentiated C2C12 cells were therefore treated with CAPE (50 μM) or with vehicle (0.1% DMSO) alone for 18 h prior to performing a 3H-deoxyglucose uptake assay in the absence of insulin. CAPE was found to increase basal rate of uptake by 225% (Fig. 1). This effect was 5.7-fold greater than the effect of 100 nM of insulin applied to vehicle-control cells 30 min prior to the uptake assay. The effect of CAPE was also superior to that of Metformin, which typically only induces a 25–40% increase in uptake following an 18 h treatment in C2C12 muscle cells [19], [20], [21] and [24].

CAPE is an uncoupler

The effects of CAPE on the respiration of isolated rat liver mitochondria were assessed in order to test the hypothesis that the reported activation of AMPK by CAPE [17] and the remarkable increase in basal glucose uptake observed above were the result of a metabolic stress induced by the disruption of energy transduction pathways. This hypothesis was appropriate in light of the known effects of several naturally occurring compounds on mitochondrial oxidative phosphorylation [13], [16], [25] and [26]. CAPE (50 μM) was observed to completely uncouple mitochondrial oxidative phosphorylation, whereby the rate of basal oxygen consumption in CAPE-treated mitochondria was increased approximately 4.5-fold, to the same rate as that achieved with ADP stimulation in vehicle-treated mitochondria, and the addition of ADP to CAPE-treated mitochondria did

not further increase oxygen consumption (Fig. 2). This uncoupling effectively abolished ATP synthetic capacity.

Effect of caffeic acid derivatives on mitochondrial function and glucose uptake

In order to assess whether other compounds related to CAPE possess similar activities and to elucidate a structure–activity relationship, twenty compounds were tested for glucose-uptake stimulating activity and for mitochondrial uncoupling activity. These compounds are illustrated in Fig. 4, functionally grouped to address discrete structural hypotheses. Activities of these compounds, in addition to some physicochemical properties, are summarized in Table 1.

A first step consisted of testing the root compound, caffeic acid; it was found to be inactive in both assays. Next, a more closely related compound, CAME, was found to increase glucose uptake by 65% and to mildly uncouple oxidative phosphorylation by 7%. In light of this finding, other caffeic acid esters were tested, including CAEE, CAAE, and CAOE; all three were found to be active, increasing uptake by 72–230%, and uncoupling oxidative phosphorylation by 14–92%.

Working from the active CAME, four closely related esters differing only in the number or position of hydroxyl substituents around the phenolic ring were tested. All were found to be inactive in either assay. Similarly, ferulic acid methyl ester was inactive. Still working from CAME, a related compound missing the characteristic double bond of caffeic acid, caffeic acid dihydro methyl ester, was found inactive; similarly, other caffeic acid dihydro esters were inactive. The compound propenyl catechol, a truncated caffeic acid devoid of the carboxylic acid ester, was also inactive. Finally, some free caffeic acids (i.e. carboxyl substituted), including rosmarinic and chlorogenic acid, were found inactive.

Effect of active caffeic acid derivatives on cellular viability

The four newly identified active caffeic acid derivatives were tested for CAPE-like cytotoxicity. CAAE, CAAE, and CAOE all decreased viability by 3–16% (Table 1). CAME, however, did not affect the release of LDH, as compared to vehicle alone.

Relationship between uncoupling of oxidative phosphorylation and stimulation of glucose uptake

Of the 21 compounds in the test set, only the five caffeic acid esters were found to induce an uncoupling effect in isolated mitochondria. These five were also the only compounds to induce an important (>30%) enhancement of glucose uptake. A linear regression analysis of the 21 compounds supported that uncoupling activity, measured as an instantaneous effect, and stimulation of glucose-uptake following an 18 h treatment, were related activities (Fig. 5). However, the closeness of fit was slightly reduced by CAOE, equally potent to CAPE at stimulating glucose uptake but a less powerful uncoupler under the conditions used here.

Stimulation of the AMPK pathway by active caffeic acid derivatives

Western immunoblot analyses were performed in order to confirm that the four newly identified active caffeic acid derivatives activated the AMPK pathway, as has been reported for CAPE. The content of phosphorylated ACC, an effector of AMPK, was assessed in C2C12 cells treated with caffeic acid derivatives (50 µM) or with vehicle alone for 18 h. All four compounds and CAPE were found to induce a long-lived phosphorylation of ACC, although in all cases the content of phospho-ACC was inferior to that induced by the AMP mimetic AICAR (1 mM) applied to vehicle-control cells over the last 30 min of treatment (Fig. 6).

Relationship between lipophilicity and effect on mitochondrial function

An important physicochemical property that varied between the five active caffeic acid derivatives was lipophilicity. Non-linear regression analyses were therefore performed to assess whether lipophilicity was a predictor of activity. Lipophilicity, expressed as the log of the predicted octanol–water partition coefficient (P), was found to be well-related ($r2 = 0.99$) to enhancement of glucose uptake by a sigmoidal dose–response function (Fig. 7A). Lipophilicity was also found to be well-related ($r2 = 0.99$) to the decrease of cellular

viability, again by a sigmoidal function (Fig. 7B). Finally, lipophilicity was well-related ($r2 = 0.99$) to uncoupling over the log P range of 1.9–3.9, also by a sigmoidal function (Fig. 7C); CAOE, the most lipophilic compound, induced less uncoupling than CAPE or CAAE under the conditions used here, as noted above and as discussed below.

Discussion

Derivatives of caffeic acid, a subtype of cinnamic acid, are widely distributed in the plant kingdom and are found in coffee beans, wheat, oat, and several fruits and vegetables. They typically occur not as free acids, but as esters, amides, and glycosides, or as dimers and other more complex forms [27]. These compounds as a family have received much attention in recent years and a variety of activities, including anti-bacterial, anti-cancer, anti-inflammatory, anti-atherosclerotic, anti-oxidant, immunomodulatory and neuroprotective, have been ascribed to them [28], [29], [30], [31], [32], [33], [34], [35], [36], [37], [38], [39], [40], [41] and [42]. It has also been proposed that caffeic acid derivatives may possess anti-diabetic activities [43], [44], [45] and [46]. A particularly interesting member of this family is caffeic acid phenethyl ester (CAPE), best known as one of the main botanical components of honeybee propolis [47], a glue-like substance used in the making of beehives and which exhibits potent anti-microbial activity believed to contribute to the aseptic environment of the hive. CAPE has recently been observed to exhibit anti-diabetic activity in the form of potent AMPK-mediated stimulation of glucose uptake in skeletal muscle cells [17]. As AMPK is considered a key therapeutic target for metabolic diseases and current therapies for exploiting this target are limited, the present study was designed to probe the potential of CAPE and caffeic acid derivatives as novel activators of AMPK with glucose-uptake stimulating activity. Specifically, the aims of the study were to confirm the effect of CAPE, elucidate the mechanism by which it activates AMPK, identify other caffeic acid derivatives with similar anti-diabetic activity, elucidate the structural components and physicochemical properties essential to their activity, and evaluate the relationship between activity and cytotoxicity.

Lee et al. [17] have recently shown that, following a 1 h treatment, CAPE induces an AMPK-mediated stimulation of glucose uptake in L6 skeletal muscle cells that is comparable to the effect of 100 nM insulin. In the present study, we report that a treatment duration of 18 h at 50 μM resulted in more than a 3-fold increase in basal (non-insulin-stimulated) glucose uptake in C2C12 muscle cells, an effect approximately 6-fold that of 100 nM insulin applied acutely. This larger effect magnitude relative to the Lee et al. study [17] may be due to our use of longer treatment duration and of a less insulin-responsive cell

line, both factors permitting a better appreciation of the contribution of transcriptionally mediated effects rather than effects only at the level of translocation and activation of glucose transporters. Indeed, increased expression of effector proteins, such as the glucose transporter GLUT4, appears a likely explanation for effects surpassing those achieved acutely with a pharmacological dose of insulin and presumably representing the system's maximal capacity. If a long-lived translocation effect contributed to some of the observed stimulation, as may be suggested by the finding that the AMPK effector ACC remains phosphorylated at the end of the 18 h treatment, this contribution would be expected to be on the order of the acute effect of insulin since AMPK-induced transporter translocation is mediated by signaling events that converge with the insulin receptor pathway. Similarly as shown by Lee et al. [17], a contribution of the insulin receptor pathway to the effect of CAPE was excluded by the demonstration that there was no increase in the phosphorylation of Akt, a downstream marker involved in glucose transporter translocation, coinciding with the enhancement of glucose uptake (not shown). The exceptionally large increase in glucose uptake can therefore best be explained by other mechanisms attributed to AMPK, namely an increase in maximal capacity for glucose uptake [6], [7], [22] and [23].

To test the hypothesis that CAPE activates AMPK by disrupting mitochondrial function and producing metabolic stress, CAPE was applied to isolated rat liver mitochondria and its effects on oxygen consumption were monitored. Our results demonstrate that CAPE at 50 μM induced complete uncoupling of liver mitochondria, whereby the rate of basal (i.e. State 4) oxygen consumption was immediately and irreversibly increased above that which can be achieved by ADP, and the addition of ADP had no further effect. These results will be further strengthened by assessing the effects of CAPE in skeletal muscle's two distinct mitochondrial populations. Because of the magnitude of CAPE's effect on mitochondrial function, its cytotoxicity was assessed by LDH assay in C2C12 cells; CAPE was observed to reduce viability by 14% following an 18 h treatment at 50 μM (Fig. 3). Our finding that CAPE exhibits a potent uncoupling effect and can completely dissipate ATP synthetic capacity in isolated mitochondria concords with its use as an anti-microbial compound by plants and insects, and with other botanical components of propolis exhibiting uncoupling activity [16]. This finding is also in accord

with the activation of AMPK being the result of a metabolic stress [8] and [48]. Although the uncoupling-type disruption of mitochondrial function observed here is different from the inhibition of complex I of the electron transport chain that is induced by Metformin [49] and [50], the proposed mechanism of action by which CAPE indirectly induces the activation of AMPK is nevertheless analogous to that of Metformin.

Caffeic acid esters closely related to CAPE were tested for glucose uptake stimulating activity and for mitochondrial uncoupling activity. These included caffeic acid methyl ester (CAME), caffeic acid ethyl ester (CAEE), caffeic acid diallyl ester (CAAE), and caffeic acid n-octyl ester (CAOE). All four were found to potently increase basal uptake by 65–230% when applied at 50 μM for 18 h. These same four were also found to uncouple mitochondria by 7–92%. Finally, these compounds were tested for cytoxicity and it was found that, with the exception of CAME, they reduced viability by 3–16%. Stimulation of glucose uptake and mitochondrial uncoupling have never been attributed to these well-known compounds, although CAOE, like CAPE has received attention for its anticancer activity [51], [52] and [53], a property that could be related to the effect on mitochondrial function and the subsequent activation of AMPK, observed herein; indeed, cellular proliferation is one of the many synthetic processes that are acutely inhibited by AMPK [54].

Sixteen other related compounds were selected to address specific structure–activity hypotheses. Of these, none exhibited uncoupling activity nor significantly stimulated muscle cell glucose uptake. The finding that caffeic acid and other related free acids were inactive suggests that a carboxyl group and perhaps other strongly ionizable substituents are incompatible with activity, possibly due to decreased membrane permeability of ionized compounds. The requirement for an intact catechol moiety was revealed by an absence of activity in compounds related to CAME but differing in the number or position of hydroxyl substituents. Similarly, absence of the caffeic acid double bond or of the carboxylic acid ester was also found to abolish activity. The structural elements occurring beyond the ester can therefore be considered as a single substituent not essential to activity but whose nature modulates activity. As such, it can be predicted that caffeic acid esters composed of a large

variety of “substituents” not containing a strongly ionizable group should be active. This is summarized in Fig. 8.

Uncoupling is defined herein as an increase in respiration (i.e. substrate oxidation) with no commensurate increase in the synthesis of ATP through oxidative phosphorylation. This increase in oxygen consumption is a reflection of an increase in the pumping rate of protons out of the mitochondrial matrix to compensate for an induced proton influx or “leak”. The leak and the compensatory pumping of protons therefore amount to a futile metabolic cycle. The increase in oxygen consumption represents a portion of mitochondrial respiratory capacity diverted to countering the leak, and therefore a corresponding decrease in the maximal rate of ATP synthesis. AMPK can be expected to be activated as a result of the increased work performed pumping protons and of insufficient residual mitochondrial capacity to meet the cell's energy needs (i.e. metabolic stress). An uncoupling effect can be induced by any of a number of protonophoric mechanisms, the best studied of which involves the shuttling of protons across the inner mitochondrial membrane by lipophilic weak acids that diffuse into the mitochondrial matrix in neutral form, release a proton, and diffuse back to the mitochondrial intermembrane space (IMS) in ionized form [55] and [56]. Protons can be similarly shuttled by certain fatty acids that intercalate inner mitochondrial membrane phospholipids and exist there in both neutral and ionized forms [56] and [57]. The caffeic acid derivatives exhibiting uncoupling activity are unlikely to be acting as proton shuttles since, by virtue of their pKa on the order of 9.2, they are expected to exist predominantly in the neutral form at mitochondrial matrix pH (approximately 8.0), and almost exclusively in this form at mitochondrial IMS pH (approximately 7.4). These compounds may therefore be indirect protonophores rather than shuttles. One possibility is that the compounds interact with a transmembrane protein that can increase proton conductance. Such proteins include transporters like the adenosine nucleotide transporter, the aspartate/glutamate transporter, and the dicarboxylate carrier, either alone or as part of the mitochondrial permeability transition pore (MPTP) or another complex [56], [58] and [59]. The potential interaction of caffeic acid derivatives with a protein is supported by the observation that activity is conferred only by a very specific structure (i.e. the caffeic acid moiety) and that small deviations in this structure abolish activity, as discussed above. In

contrast, proton shuttles are not subject to such severe structural constraints, but rather to constraints at the level of their physicochemical properties [55] and [56]. The interaction of resveratrol and of quercetin with ATP synthase, recently elucidated by crystallography [15], constitutes a precedent for binding of naturally occurring small phenolic compounds to protein components of the oxidative phosphorylation system. Interaction with a protein of the MPTP, and subsequent proton conductance through the pore, has been proposed by others to explain the uncoupling activity of curcumin [60] and [61], a compound closely related to caffeic acid derivatives. Based on the structural constraints for activity, it can be speculated that the proposed interaction is mediated by the two hydroxyl substituents of the catechol moiety in addition to the carboxylic acid ester, that the ester must be coplanar with the phenolic ring by virtue of the double bond, and that the interaction occurs on the matrix side of the inner mitochondrial membrane, inaccessible to negatively charged compounds.

The results demonstrate that whereas an intact caffeic acid moiety is essential for activity, the “substituent”, or structure occurring after the carboxylic acid ester, can assume a variety of forms. However, despite not being subject to stringent structural constraints, this portion can nevertheless greatly affect the properties of the entire compound. From a physicochemical perspective, the most important difference between the five active compounds tested here is their lipophilicity as the compounds span a predicted log P range of 1.9–5.0. Interestingly, this property was found to be a strong predictor of activity, with goodness of fit coefficients (r2) of ≥0.99 observed between log P and both glucose uptake and cytotoxicity when these relationships were modelled by sigmoidal dose–response functions. It can therefore be expected that the activity of other active caffeic acid esters will be found to be also predicted by lipophilicity. It must be noted that a strong sigmoidal relationship between lipophilicity and mitochondrial uncoupling activity was only observed within the log P range of 1.9–3.9. The most lipophilic compound, CAOE, exhibited less uncoupling activity than either CAPE or CAAE. Modest activity in highly lipophilic compounds could be explained by a decrease in effective concentration due to the phenomenon of membrane retention. However, in the present case, it may be explained by an underestimation of uncoupling activity, suggested by a significantly greater decrease in residual mitochondrial capacity than can be accounted for by the uncoupling effect alone.

Such concurrent inhibition of respiration has been suggested by others to be due to inhibition of the mitochondrial transport of succinate [62], [63] and [64], the substrate used in our isolated mitochondrial preparations. Interestingly, CAPE exhibits a similar effect at concentrations greater than the 50 μM used in the present study (data not shown). In whole cells, mitochondria oxidize other substrates in addition to succinate, and therefore respiration would not be expected to be severely compromised by a partial inhibition of succinate transport. It is therefore possible that the true uncoupling activity of CAOE may be similar to that of CAPE, just as the glucose-uptake stimulating activity of these compounds is closely matched. In this case, the relationship between lipophilicity and uncoupling activity would mirror the observed relationship between lipophilicity and stimulation of glucose uptake.

While the disruption of mitochondrial function through which caffeic acid derivatives promote the activation of AMPK is slightly different from the inhibitory-type disruption induced by the biguanides, as discussed above, both mechanisms can potentially cause lactic acidosis. Therefore, as is the case for the biguanides, the safety of caffeic acid derivatives can only be maximized at the cost of activity. However, results indicate that some of the active caffeic acid derivatives identified here exhibit only a small potential for toxicity while still inducing significant stimulation of glucose uptake and prolonged phosphorylation of ACC. This is especially true of CAME as its small uncoupling effect (7% at 50 μM) translates into a useful increase in glucose uptake (65% at 50 μM; more than 1.5-fold the effect of insulin), without negatively impacting cell viability. However, even the more powerful compounds of the test group that completely or almost completely compromise mitochondrial ATP synthesis, only reduced viability by up to 16%. One way to reconcile these findings is to suggest that their effect on mitochondria is short-lived. It is indeed known that phenolic compounds with hydroxyl substituents can undergo rapid glucuronidation [65]. In the case of active caffeic acid derivatives, such glucuronidation would likely render compounds inactive toward mitochondrial uncoupling. Whereas such short-lived mitochondrial activity would suggest equally short-lived metabolic stress and activation of AMPK, the downstream effects of AMPK, including its effects on gene

expression, are longed-lived. It may therefore not be necessary nor desirable to prolong metabolic stress.

The very promising ratio of activity to cytotoxicity of CAME warrants further study into the potential of this compound and of other related derivatives as treatments for insulin resistance and suggests that activation of AMPK through disruption of mitochondrial function need not have a narrow margin of safety.

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Figure Legends

Figure 1. Caffeic acid phenethyl ester (CAPE) increased non-insulin-stimulated (basal) 3H-deoxyglucose uptake in differentiated C2C12 skeletal muscle cells by more than 3-fold. Cells were treated with 50 µM of CAPE or with vehicle (0.1% DMSO) for 18 h. The effect of CAPE was 5.7-fold greater than that of 100 nM insulin applied acutely for the last 30 min of the treatment in vehicle-treated cells. Data are expressed normalized to basal uptake of the vehicle control group. Data are presented as the mean of 3 experiments ± SEM, each experiment composed of 3–4 replicates per condition. * Indicates a significant ($p \leq 0.05$) difference from the vehicle control group, as assessed by ANOVA.

Figure 2. CAPE induced a powerful uncoupling effect in isolated rat liver mitochondria. Representative tracings of succinate-supported basal (State 4) and ADP-stimulated (State 3) O2 consumption, assessed at 25 °C with a Clark-type oxygen electrode. The uncoupling effect of CAPE at 50 µM (dark tracing) was complete in that the rate of basal O2 consumption was increased to slightly more than the rate of ADP-stimulated O2 consumption in vehicle-treated mitochondria (light tracing) and the addition of ADP did not further increase O2 consumption. Values represent rate of consumption in nmoles O2 per mg mitochondria per minute.

Figure 3. CAPE induced cytotoxicity in C2C12 myotubes. Cytotoxicity was assessed by the release of lactate dehydrogenase (LDH) into the cell medium over an 18 h treatment with 50 µM CAPE or vehicle (0.1% DMSO). Released LDH was expressed as a % of total LDH. Data are presented as the mean of 3 experiments ± SEM. * Indicates a significant ($p \leq 0.05$) difference from the vehicle control group.

Figure 4. Compounds selected to address specific structure–activity hypotheses. Compounds were initially selected based on a relation to CAPE. Further testing was performed on compounds more closely related to CAME. Each arrow represents a specific hypothesis. Arrows with crosses indicate that the respective derivatives, and any nested derivatives, are inactive.

Figure 5. The magnitude of stimulation of glucose uptake in C2C12 mytotubes induced following an 18 h treatment with caffeic acid derivatives is correlated to the magnitude of mitochondrial uncoupling activity induced by these compounds in isolated liver mitochondria. Data are presented as mean ± SEM.

Figure 6. Active caffeic acid derivatives increased phosphorylation of ACC, an effector of AMPK, in C2C12 myotubes. Shown are representative immunoblots of cells treated for 18 h with either vehicle (0.1% DMSO; lane 1) or 50 μM of the various compounds (lanes 3–7). The upper blot was probed with anti-phospho-ACC. The lower blot was probed with anti-β-actin as a control. AICAR (1 mM) applied acutely for the last 30 min of the treatment in vehicle-treated cells was used as positive control (lane 2) for the activation of the AMPK pathway.

Figure 7. The lipophilicity of active caffeic acid derivatives is a good predictor of their activity. The stimulation of muscle cell glucose uptake (A) as well as the negative impact on C2C12 viability (B) were well-related to estimated lipophilicity, expressed as log of the octanol–water partition coefficient (P). Mitochondrial uncoupling (C) was also well-related to lipophilicity, but only over a log P range of 1.9–3.9; the uncoupling activity of the most lipophilic compound CAOE was determined to be smaller than that of CAPE and CAAE and may have been underestimated due to concurrent inhibition of mitochondrial respiration.

Figure 8. Structural constraints for bioactive caffeic acid derivatives. Activity requires that: (1) the catechol moiety be intact; (2) the double bond between the first and second carbons of the side chain be present; (3) the carboxylic acid ester be present; (4) the side chain not contain strongly ionizable groups. Beyond these constraints, the side chain can be composed of various structures, modulating activity in accordance with the lipophilicity that they confer upon the compound; within the log P range of 2–5, activity is linearly related to lipophilicity.

Table 1 Physicochemical parameters and measured activities of test compounds

Compound	Predicted pKa	Predicted logP	Isolated Mitochondria		Skeletal Muscle Cells	
			Uncoupling Effect (50 µM)	**Residual Capacity** (50 µM)	**Δ Glucose Uptake** (50 µM)	**Δ Viability** (50 µM)
caffeic acid	3.1; 9.3; 12.7	1.5	-1 % ± 1 %	90 % ± 4 %	+4 % ± 9 %	
caffeic acid, dihydro ethyl ester	9.3; 12.7	2.0	0 % ± 0 %	115 % ± 13 %	+ 12 % ± 5 %	
caffeic acid, dihydro methyl ester	9.3; 12.7	1.6	0 % ± 1 %	104 % ± 3 %	+28 % ± 5 %	
caffeic acid, dihydro phenethyl ester	9.3; 12.7	3.6	2 % ± 1 %	80 % ± 14 %	+13 % ± 7 %	
caffeic acid, ethyl ester (CAEE)	9.2; 12.6	2.3	14 % ± 1 %	86 % ± 10 %	+72 % ± 6 %	-3 % ± %
caffeic acid, methyl ester (CAME)	9.2; 12.6	1.9	7 % ± 4 %	85 % ± 9 %	+65 % ± 12 %	0 % ± %
caffeic acid, n-octyl ester (CAOE)	9.2; 12.6	5	48 % ± 1 %	18 % ± 3 %	+230 % ± 20 %	-16 % ± %
caffeic acid, phenethyl ester (CAPE)	9.2; 12.6	3.9	105 % ± 20 %	0 % ± 0 %	+225 % ± 21 %	-14 % ± %
caffeic acid, 1,1-dimethylallyl ester (CAAE)	9.2; 12.6	3.3	92 % ± 10 %	3 % ± 3 %	+158 % ± 15 %	-9 % ± %
chlorogenic acid	3.3; 9.2; 12.5	-0.3	2 % ± 1 %	105 % ± 3 %	-6 % ± 7 %	
cinnamic acid	4	2.1	1 % ± 0 %	93 % ± 5 %	+4 % ± 2 %	
cinnamic acid, 2,4-dihydroxy methyl ester	8.7; 10.7	1.9	1 % ± 0 %	97 % ± 1 %	-7 % ± 6 %	
cinnamic acid, 2,5-dihydroxy methyl ester	9.5; 11.3	1.9	-1 % ± 1 %	90 % ± 13 %	+4 % ± 6 %	
cinnamic acid, methyl ester	n/a	2.5	0 % ± 1 %	87 % ± 1 %	+14 % ± 6 %	
cinnamic acid, 4-hydroxy methyl ester	9.4	2.2	1 % ± 0 %	104 % ± 8 %	+2 % ± 4 %	
ferulic acid	3.3	1.7	0 % ± 0 %	108 % ± 8 %	-6 % ± 7 %	
ferulic acid, ethyl ester	9.9	2.4	1 % ± 2 %	104 % ± 7 %	-10 % ± 5 %	
ferulic acid, methyl ester	9.9	2.1	0 % ± 0 %	93 % ± 8 %	+11 % ± 13 %	
ferulic acid, phenethyl ester	9.9	4.1	0 % ± 1 %	87 % ± 1 %	-68 % ± 2 %	
4-(1-propenyl)-catechol	9.3: 12.7	2.5	0 % ± 1 %	107 % ± 5 %	+11 % ± 3 %	
rosmarinic acid	3.1	3	0 % ± 1 %	103 % ± 3 %	+6 % ± 5 %	

Notes:
pKa = acid dissociation constant; P = octanol-water partition coefficient for the neutral form of compound; n/a = not applicable.
Data are expressed as mean ± SEM.
Respiration data were collected from 2 separate experiments performed in duplicate.
Calculations of Uncoupling Effect and of Residual Mitochondrial Capacity are described under Methods.
Residual Mitochondrial Capacity is the net result of the Uncoupling Effect and any inhibition of respiration or of ATP synthase
Glucose uptake data are expressed relative to the vehicle control group (SEM = 8 %) and were collected from 3 separate experiments performed in triplicate. Treatment duration was 18 h.
Viability was calculated from % of total LDH released into the medium.
Viability data are expressed relative to vehicle control group (SEM = %) and were collected from 2 separate experiments performed in triplicate. Treatment duration was 18 h.

Figures

Figure 1

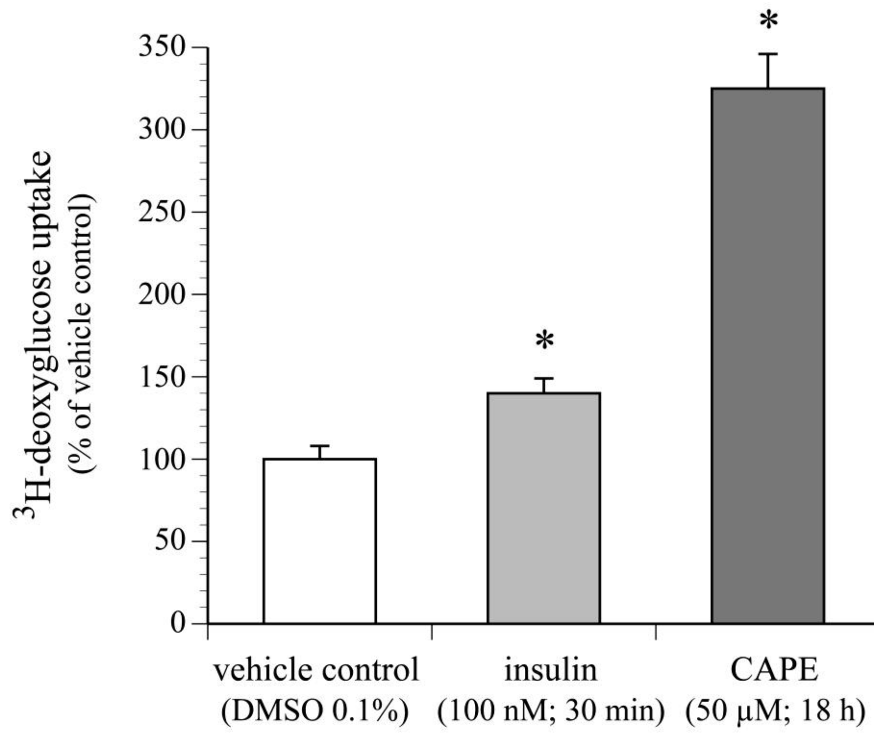


Figure 2

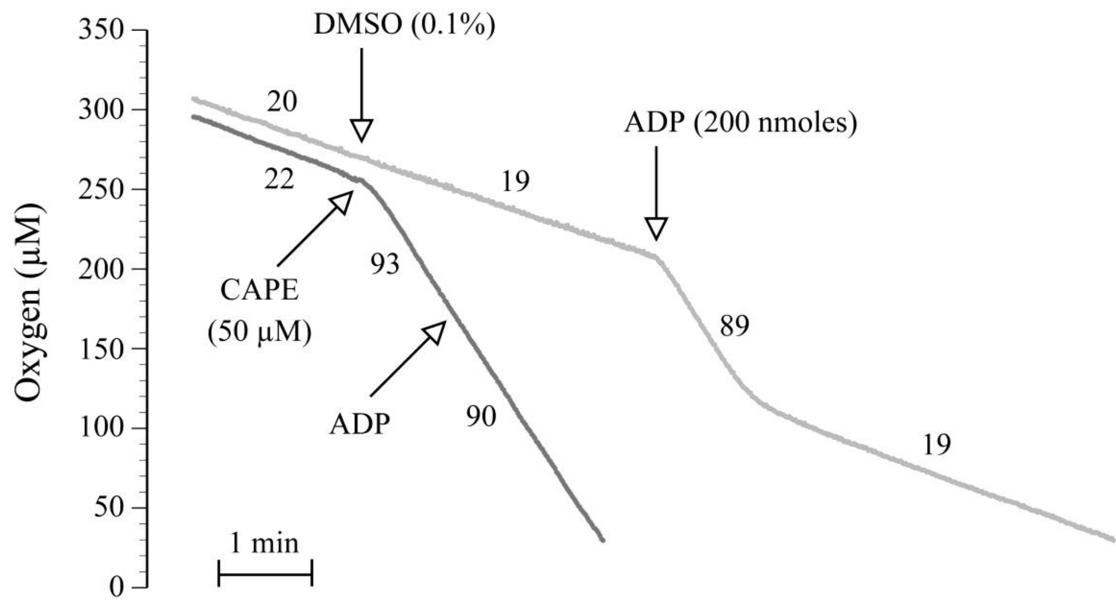


Figure 3

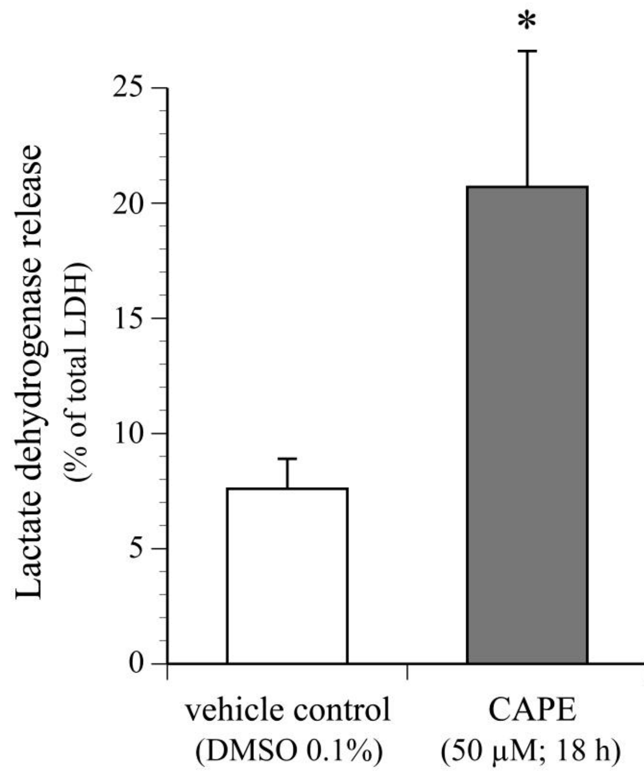


Figure 4

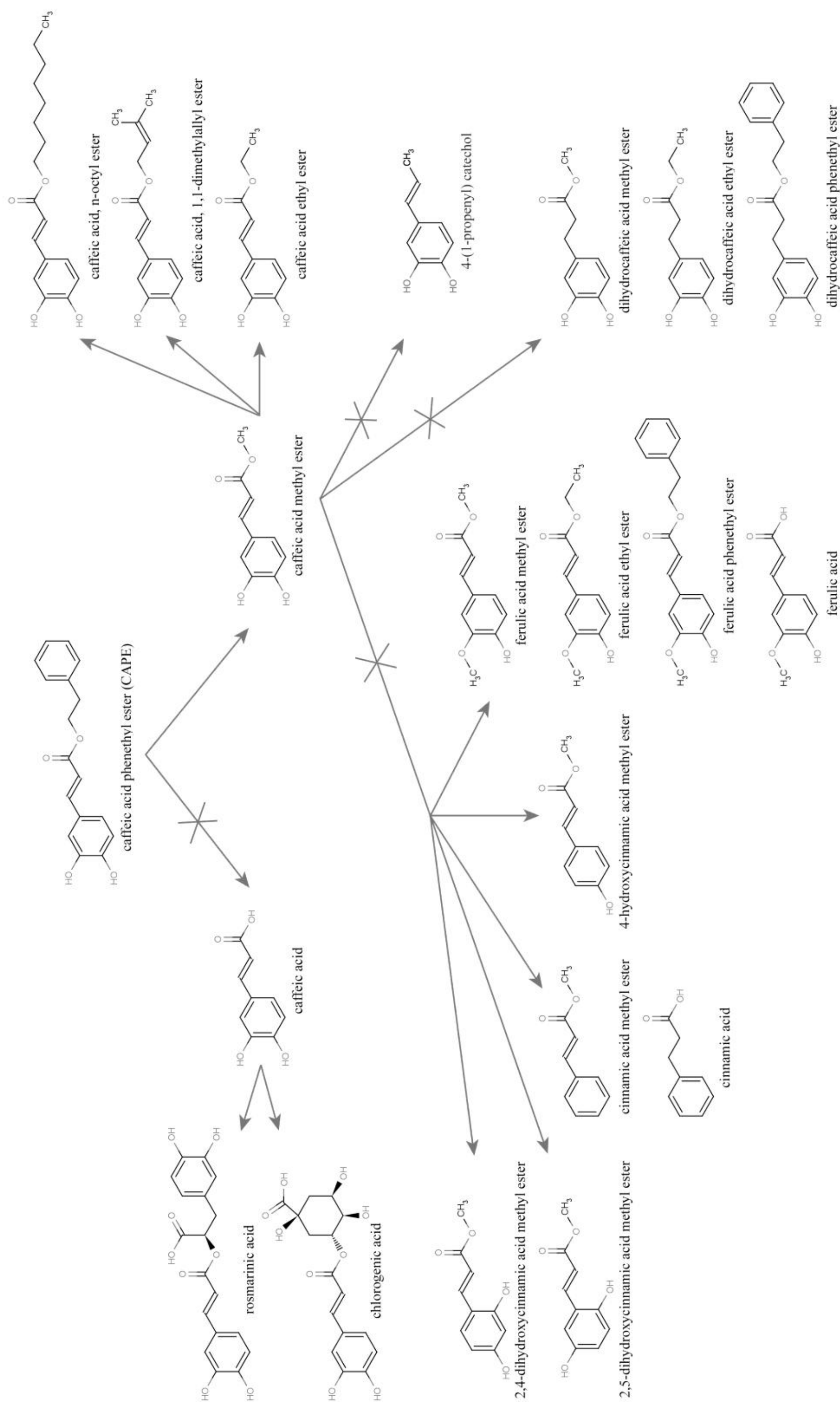


Figure 5

r^2=0.80

% mitochondrial uncoupling

100
80
60
40
20
0

0 50 100 150 200 250

% increase
glucose uptake

Figure 6

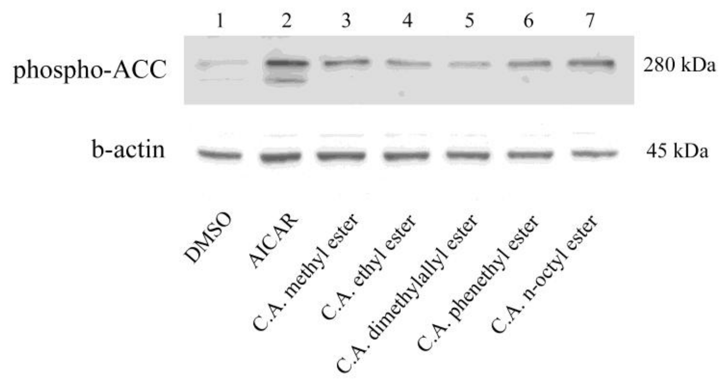


Figure 7

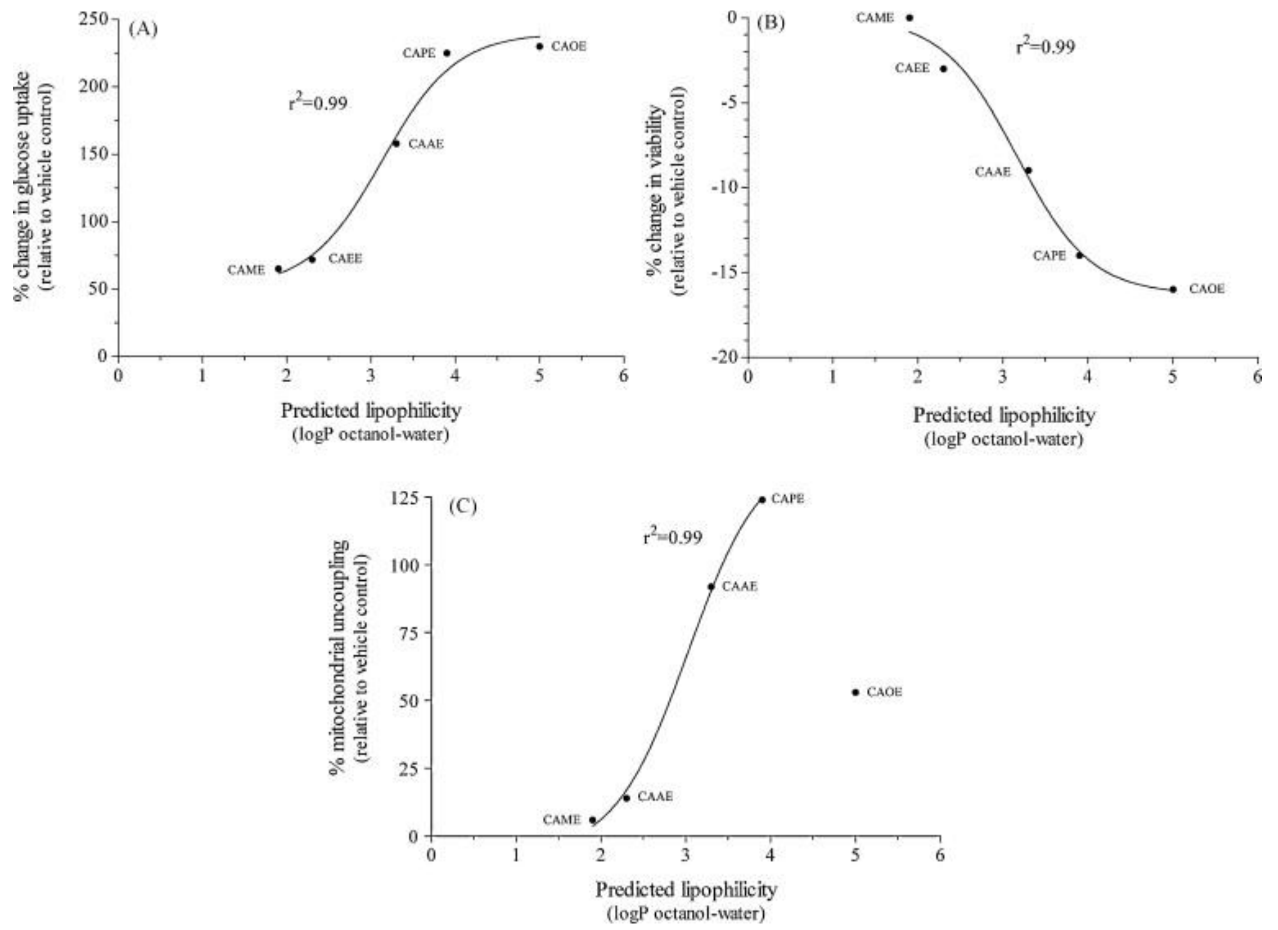
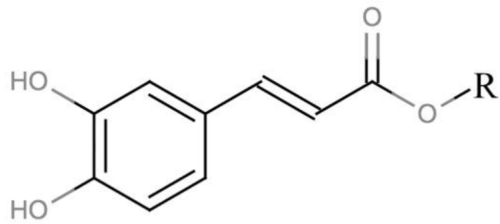


Figure 8



where R:
1) confers logP ≥ 2
2) contains no ionizable group with pKa < 9

4. Article 3

Vaccinium vitis-idaea, a medicinal plant of the Eastern James Bay Cree, mobilizes L6 muscle Glut4 transporters and exerts anti-obesity and antidiabetic effects *in vivo*.

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Authors' contribution

I performed the experimental work, data analysis and wrote the paper.

Antoine Brault contributed to the *in vivo* study and data analysis.

Meriem Ouchfoun and Diane Vallerand contributed to the *in vivo* study.

Farah Thong helped with translocation of GLUT4 transporters assay which I performed in Dr Gary Sweeney laboratory and she reviewed the paper.

Dr Pierre Haddad is my supervisor.

The crude plant extract was prepared in the laboratory of Dr John Arnson.

Keywords: type 2 diabetes mellitus, GLUT4, OPD, KKA^y mice, ACC, AMPK, pair-feeding, PPAR-α, natural health products, traditional medicine, Canadian boreal forest, Aboriginal populations of North America.

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Abstract

Objective: *Vaccinium vitis idaea* (mountain cranberry) has been identified among species used by the Cree of Eeyou Istchee of northern Quebec to treat symptoms of diabetes. In a previous study, the ethanol extract of the berries was found to enhance glucose uptake in C2C12 muscle cells through stimulation of AMP-activated protein kinase (AMPK) pathway (Eid et al., 2010). In this study, we investigated the effect of this product on the translocation of insulin-sensitive glucose transporters GLUT4 in skeletal muscle cells in culture. To validate the effect of *V. vitis in vivo*, the extract was administered to diabetic animals.

Methods: L6 cells were treated with *V. vitis* (200 μg/ml) for 18 h. For the *in vivo* studies, *V. vitis* (1%) in drinking water was administered to KKA^y mice for 10 days.

Results: *V. vitis* significantly increased glucose uptake and GLUT4 translocation to the cell membrane of L6 cells. The extract increased phosphorylation of AMPK and p38 MAPK with no indication of increased phosphorylation of Akt. *V. vitis* (1%) in drinking water administered to KKA^y mice for 10 days decreased glycemia by 32%. Cumulative food and fluid intakes and body weight were also significantly reduced by *V. vitis*. Moreover, *V. vitis* treatment increased expression of GLUT4 in skeletal muscle and stimulated the phosphorylation of ACC and increased the levels of PPAR-α in the liver of KKA^y mice.

Conclusion: *V. vitis* may improve hyperglycemia by promoting GLUT4 translocation in L6 skeletal muscle cells through an insulin-independent mechanism involving AMPK. The *in vivo* animal studies showed that *V. vitis* exhibited significant anti-hyperglycemic and anti-obesity effects in diabetic KKA^y mice due to appetite reducing properties. The results of the present study confirm the potential of *V. vitis* berries for the prevention and treatment of obesity and diabetes.

Introduction

Central obesity and insulin resistance are the cornerstones of the metabolic syndrome, a cluster of three or more abnormalities that includes central adiposity, hypertriglyceridemia, elevated low density lipoprotein, reduced high density lipoprotein cholesterol, glucose intolerance and hypertension (Cohn et al., 2001). The metabolic syndrome is more prevalent in females and certain ethnic groups, particularly aboriginal populations (Pollex et al., 2006). It is now well established that central obesity, measured notably by elevated waist circumference, increases the risk for development of type 2 diabetes and cardiovascular disease (Haffner, 2006). Indeed, obesity generally leads to insulin resistance, a condition that describes the impaired ability of cells to respond to insulin in promoting its various biological actions. Insulin resistance affects glucose transport into skeletal muscle and other insulin sensitive tissues, a process mediated by the translocation of GLUT4 glucose transporter to the cell membrane. Insulin stimulates GLUT4 translocation in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner (Wang et al., 1999). In addition, GLUT4 translocation is also regulated by the insulin-independent AMP-activated protein kinase (AMPK) pathway. AMPK acts as the intracellular energy sensor and is activated under conditions of metabolic stress (Holmes et al., 1999), (Kurth-Kraczek et al., 1999), (Hayashi et al., 2000).

As a consequence to decreased insulin-mediated glucose uptake in skeletal muscle and adipose tissue, a compensatory hyperinsulinemia develops to prevent the appearance of frank hyperglycemia. When β-pancreatic cells cannot maintain the amount of insulin required to overcome the resistance, type 2 diabetes develops. Several studies also reported that primary hyperinsulinemia rather than compensatory hyperinsulinemia might be the primary genetic defect in some ethnic groups with a high prevalence of diabetes, such as Native Americans, Mexican-Americans and Pacific Islanders (Weyer et al., 2000). Aboriginal populations are particularly at risk for developing type 2 diabetes mellitus and its complications. In Canada, the prevalence of diabetes for these populations is at least three times higher than that of the general population and is expected to increase three-fold over the next 20 years (Young et al., 2000).

Vaccinium vitis idaea is a medicinal plant used by Cree communities of Eeyou Istchee (CEI, Eastern James Bay region of the Canadian province of Quebec) to treat several symptoms of diabetes (Leduc et al., 2006), (Fraser et al., 2007). This plant was identified by our research team during a previous bioactivity screening study, as a part of a project aiming to provide culturally relevant alternative treatment options for Cree diabetics, whose disease prevalence is among the highest in Canada (Harbilas et al., 2009). The genus *Vaccinium* includes various members reputed to possess antidiabetic activity and are traditionally used for the treatment of diabetes by several cultures throughout the world, e.g. lowbush blueberry (*V. angustifolium*), American cranberry (*V. macrocarpon*) and European bilberry (*V. myrtillus*) (11).

Since insulin resistance is a major cause of type 2 diabetes, there has been growing interest in insulin sensitizers for the treatment of this disease. The two most commonly used antidiabetic drugs are thiazolidinediones (TZDs) and biguanides. The main side effects of TZDs are weight gain and fluid retention. The biguanide metformin acts mainly on the liver to suppress hepatic glucose production and does not cause weight gain but might be associated with lactic acidosis (Baba et al., 2001), (Vasudevan and Balasubramanyam, 2004), (Zangeneh et al., 2003), (Krentz and Bailey, 2005).

In our previous study, *V. vitis* was found to increase glucose transport in muscle cells through the activation of AMPK as a response to metabolic stress resulting from a non-toxic disruption of mitochondrial energy transduction (Eid et al., 2010). The present study was carried out firstly to determine whether *V. vitis* increases GLUT4 translocation in skeletal muscle cells as suspected from enhanced glucose transport. We selected L6 myocytes because they express more Glut4 proteins than our previous C2C12 cellular model and because tools exist to better ascertain Glut4 translocation to the plasma membrane. Secondly, our objective was to evaluate the antidiabetic activity of *V. vitis* in an *in vivo* model of type 2 diabetes. KKA^y mice are a cross between glucose-intolerant black KK female mice and yellow obese A^y male mice. They are characterized by hyperphagia, insulin resistance, hyperinsulinemia, diabetes, dyslipidemia and hypertension. Therefore, KKA^y mice are an excellent model for type 2 diabetes induced by obesity (Adachi et al., 2006). This model was thus selected to evaluate the *in vivo* antidiabetic activity of *V. vitis*.

Materials and methods

Plant material and extraction

Berries of *V. vitis* were collected in Whapmagoostui, QC, Canada, and kept at -20°C until use. Botanical identity was confirmed by Dr. Alain Cuerrier (Institut de recherche en biologie végétale, Université de Montréal), plant taxonomist on our Team, and voucher specimens were deposited at the Montreal Botanical Garden herbarium (voucher # Whap04-21). The 80 % ethanolic extract was prepared as previously described (Eid et al., 2010).

Cell culture

Rat L6 skeletal muscle cells were grown in minimum essential medium alpha (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a 5% CO_2 at 37°C and used as myoblasts when fully confluent. For differentiation into myotubes, cells were switched to a medium containing 2% FBS for 5–7 days. Cells transfected to stably overexpress GLUT4 harbouring a myc epitope on the first exofacial loop of the transporter (L6 GLUT4myc cells) were kindly provided by Dr Amira Klip (The Hospital for Sick Children, Toronto, ON, Canada).

Measurement of glucose uptake

L6-GLUT4*myc* cells were cultured in 12-well plates and were used after 5-7 days of differentiation. The cells were serum-starved for 4 h before being incubated with *V. vitis* (200 µg/ml) for 18 h or insulin (100 nM, 20 min). Cells were incubated in transport solution [140 mM NaCl, 20 mM HEPES-Na, 2.5 mM MgSO4, 1 mM CaCl2, 5 mM KCl, 10 µM 2-Deoxy-Glucose and 0.5 µCi/ml 2-deoxy-D-[^{3}H]glucose (pH 7.4)] for 5 min at room temperature. Cells were then lysed with 1 M KOH and aliquots were transferred to scintillation vials for ^{3}H radioactivity counting and expressed as fold increase over control. Nonspecific uptake was measured in the presence of cytochalasin B (10 µM) and was subtracted from all values.

Determination of cell surface GLUT4

Levels of GLUT4*myc* at the cell surface were measured by an antibody-coupled colorimetric assay (Niu et al., 2003). Briefly, L6 myoblasts were cultured in 24-well plates until confluence and serum-starved for 4 h before being incubated with either *V. vitis* (200 μg/ml) for 18 h or insulin (100 nM) for 20 min. Cells were then quickly washed in ice-cold PBS and incubated with an anti-c-myc antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 60 min. After that, cells were washed and fixed in 3% paraformaldehyde for 3 min on ice. To neutralize the fixative, cells were incubated with 10 mM glycine in ice-cold PBS for 10 min, and then blocked in 5% goat serum for 30 min. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were then applied for 60 min at 4°C (1:1,000 dilution; Cell Signaling Technologies, Danvers, MA). Cells were washed five times with ice-cold PBS and incubated with *O*-phenylenediamine dihydrochloride (OPD) reagent (1 ml/well) (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min. To stop the reaction, 0.25 ml of 3 M HCl was added to each well. The supernatant was collected and its absorbance was measured at 492 nm. Absorbance associated with nonspecific binding (primary antibody omitted) was used as a blank.

Western immunoblotting

The effects of plant products on insulin and AMPK signaling pathways in L6 muscle cells were assessed by western immunoblot. Cells were cultured in 6-well plates and treatments or vehicle alone (DMSO) were applied for 18 h to 5-7 day differentiated L6 cells. Twenty minutes prior to the end of the treatment, insulin (100 nM) or aminoimidazole carboxamide ribonucleotide (AICAR; 1 mM) were added to some vehicle-treated wells as positive controls. Cells were lysed in SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue), to wich protease and phosphatase inhibitors were added (1 μM Na_3VO_4, 1 μM leupeptin, 1 μM pepstatin, 1 μM okadaic acid and 1 μM PMSF), and passed through a syringe several times and heated (65 °C) for 5 min. Cell lysates were then centrifuged for 5 min (16 294 x g), and approximately 30 μg protein was separated on 10% SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. Membranes were then blocked with 3% BSA Tris-buffered saline (50 mM Tris–base, 150 mM NaCl, 1% Triton-X-100 and 1% NP-40) for 1 h. The

blots were incubated overnight at 4^0C with primary antibodies (1:1000) directed against the following proteins: phospho-AMPK, phospho-Akt, phospho-P38 and ß-actin (Cell Signaling Technologies, Danvers, MA). Membranes were then washed four times with Tris-buffered saline for 15 min each at room temperature and then incubated with HRP-coupled secondary antibody (1:10 000 Jackson Immunoresearch, Cedarlane Laboratories, Hornby, ON) for 1 h. Membranes were washed five times in wash buffer for 10 min each and proteins. Revelation was performed using the enhanced chemiluminescence and quantified by the Scion Image program (Scion Corporation, Frederick, MD, USA).

Animals and *in vivo* experimental protocols

Study #1: Effect of *V. vitis* on diabetic KKA^y mice

KKA^y mice were derived from an in-house colony established using breeding pairs obtained from Jackson Laboratory (Bar Harbor, Maine, US). Mice weighing 26-33 g were housed individually and kept for 1 week on a 12 h light-dark cycle in a temperature controlled chamber and provided a regular laboratory chow and water ad libitum. The animals were divided into two groups containing seven mice each, as follows: Group 1 diabetic mice received drinking tap water and served as controls; Group 2 diabetic mice were administered with 1% *V. vitis* in drinking water (equivalent to a dose of 4 g/ kg on the first day of treatment and 1.33 g/kg thereafter, due to a drop in fluid intake until the end of the experiment). During the ten days of treatment, the body weight, food intake, fluid intake and blood glucose level were determined on a daily basis. The non-fasting blood glucose concentration was measured using an Accu-Chek glucometer (Roche, Montreal, QC, Canada) by collecting blood from the tip of the tail vein. On the last day of treatment, the mice were anaesthetized, sacrificed and organs such as liver, skeletal muscle, kidney, epididymal fat pad, abdominal fat pad and dorsal fat pad were immediately removed and stored in a -80° C freezer until used. All experimental protocols were approved by the animal experimentation ethics committee of the University of Montreal and carried out in full respect of the guidelines from the Canadian Council for the Care and Protection of Animals.

Study #2: Pair-feeding effect in KKA^y diabetic mice

Pair feeding was employed in order to investigate to what extent the blood glucose-lowering effect observed with *V. vitis* in study #1 could be attributed to the observed reduction in food intake. Animals were allocated into two groups containing seven mice each as follows: Group 1 diabetic mice were administered with 1% *V. vitis* in drinking water; Group 2 diabetic mice received drinking tap water and were pair-fed to group 1 mice. Pair feeding was carried by measuring the food intake of the ad libitum-fed *V. vitis* treated mice every 24 h and presenting this amount of food to the pair-fed treated mice with a one-day delay. Food consumption, fluid intake and body weight were recorded three times weekly. At the end of the study, the mice were sacrificed, blood samples were obtained and tissues were harvested as described above.

Study #3: normal C57BL/6J mice

To study the effect on *V. vitis* on blood glucose levels and food intake in normal animals, normal C57BL/6J mice were housed as described before and randomly divided into two groups containing seven mice. Group 1 mice received drinking tap water and served as control; Group 2 mice were administered with 1% *V. vitis* in drinking water. Both groups were fed regular laboratory chow ad libitum. The experimental protocol lasted for 10 days and was performed as described above.

Blood parameters

Glycaemia was measured three times per week by collecting blood from the tail vein and using a commercial glucometer (Accu-Chek Roche, Montreal, Qc, Canada). Plasma insulin levels were determined by a radioimmunoassay kit (Linco Research, St-Charles, MO). Plasma adiponectin and leptin were measured by RIA (Linco Research, St-Charles, MO). The levels of serum triglycerides, cholesterol, HDL, LDL, creatinine, alkaline phosphatise, AST (Aspartate aminotransferase), ALT (Alanine aminotransferase) and LDH (lactate dehydrogenase) were measured by the Department of Biochemistry of Sainte-Justine's Children Hospital (Montreal, Qc, Canada).

Western blot for proteins involved in glucose and lipid metabolism

To study the role of *V. vitis* extract on the expression of GLUT4, muscles from individual mice were lysed in Tris buffer, pH 7.4 at 4°C, containing 20 mM Tris-HCl, 255 mM sucrose, 1 mM EDTA. For ACC and PPAR-α western blot analysis, samples of liver were homogenized in 1 ml of RIPA lysis buffer (25 mM Tris-HCl pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1% Triton-X-100, 0.1% SDS). For all samples, a protease inhibitor cocktail was added (Roche, Mannheim, Germany) as well as 1 mM phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Cells were lysed for 30 min on ice and were centrifuged at 12000 x g for 10 min. Supernatants were then stored at -80°C until analysis. Protein content was assayed by the bicinchoninic acid method standardized to bovine serum albumin (Roche, Laval, QC). Lysates were diluted to a concentration of 1 mg/ml total protein and boiled for 5 min in reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). One hundred μL of each sample were separated on 10 % polyacrylamide full-size gels and transferred to nitrocellulose membrane (Millipore, Bedford, MA). Membranes were blocked for 2 h at room temperature with Tween-20 and 5% skim milk in TBS (20 mM Tris-HCl, pH 7.6 and 137 mM NaCl). Membranes were then incubated overnight at 4ºC in blocking buffer with appropriate phospho-specific or pan-specific antibodies against ACC, GLUT4, PPAR-α and PPAR-γ at 1:200 to 1:1000. Membranes were washed 5 times and incubated 1.5 h at room temperature in TBS plus Tween 20 with anti-rabbit HRP-conjugated secondary antibodies at 1:50000 to 1:100000 (Jackson Immunoresearch, Cedarlane Laboratories, Hornby, ON). Revelation was performed using the enhanced chemiluminescence method and blue-light-sensitive film (Amersham Biosciences, Buckinghanshire, England).

Histological Analysis

The dissected liver sections were placed in the 10% formalin solution and were stained with hematoxylin phloxine saffron (HPS) by the Institut de Recherche en Immunologie et en Cancérologie (IRIC), Department of Histology (Université de Montréal, Montreal, QC, Canada). Each stained liver section was analyzed for the severity of lipid accumulation in the hepatocytes and was then scored based on the percentage of

hepatocytes that contained macrovesicular fat: namely, grade 0 (0-5%), grade 1(5-33%), grade 2 (33-66%), and grade 3 (66-100%)(Brunt et al., 1999); (Kleiner et al., 2005).

Statistical analysis

In vitro results as well quantification of western blot data for *in vivo* studies were analysed by one-way analysis of variance (ANOVA) using StatView software (SAS Institute Inc, Cary, NC), with post-hoc analysis as appropriate. Area under the curve (AUC) were calculated by using PRISM software (GraphPad, San Diego). For the *in vivo* studies, student t test for non-paired observation was used. Statistical significance was set at $p \leq 0.05$. Results are presented as the mean ± SEM for the indicated number of determinations/animals.

Results

V. vitis increases glucose uptake and GLUT4 translocation in L6 myotubes

To confirm that *V. vitis* increases glucose uptake in L6-GLUT4*myc* myotubes as it did in C2C12 cells (Eid et al., 2010), cells were treated with 200 µg/ml *V. vitis* for 18 h or 100 nM insulin for 20 min and tested for 2-deoxy-D-[^{3}H] glucose uptake. *V. vitis* stimulated glucose uptake by 65 ± 5 % (Figure 1A). In comparison, treatment with 100 nM insulin (positive control) stimulated uptake by 75 ± 13 % (Figure 1A).

Since GLUT4 translocation to the plasma membrane is an important step for glucose uptake into skeletal muscle (Zierath et al., 1996), we then examined the effect of *V. vitis* on GLUT4 translocation in L6 cells. The results of the OPD assay in L6-GLUT4*myc* myoblasts showed that *V. vitis* stimulated GLUT4 translocation in these cells by a factor of 1.8, an effect that is similar to the maximal effect of insulin (1.75-fold) (Figure 1B).

V. vitis increases the phosphorylation of AMPK and p38 MAPK in L6 myotubes

L6 myotubes were treated with *V. vitis* (200 µg/ml) or AICAR (1 mM) for 30 min. Consistent with our previous observations in C2C12 myocytes (Eid et al., 2010), the phosphorylation of AMPK was significantly increased by *V. vitis* (Figure 2). The plant extract also caused a significant increase in the phosphorylation of p-38 MAPK (Figure 2), a downstream substrate of AMPK involved in GLUT4 translocation (Cheng et al., 2006), (Somwar et al., 2001). In contrast to what was observed with the insulin positive control, *V. vitis* did not increase the phosphorylation of Akt, a downstream substrate of PI3-K and mediator of insulin signaling and GLUT4 translocation (Figure 2).

Effects of *V. vitis* on body weight, food intake, blood glucose, and fluid intake of diabetic and normal mice

In order to investigate the cumulative effect of *V. vitis* on the different parameters in time, we calculated the area under the curve (AUC). The cumulative change in body weight (BW) was significantly lower in diabetic KKA^y mice administered with *V. vitis* than in control mice receiving only drinking water (Figure 3; $p<0.05$, n=7 per group). This was correlated with significant reduction in cumulative food intake (16%) in *V. vitis*- treated group compared to vehicle control animals (Figure 3; $p<0.05$, n=7 per group). Interestingly,

daily administration of *V. vitis* to these mice resulted in a significant decrease of glycemia as compared to controls (32%, Figure 3, p<0.05, n=7 in each group).

Hence, to verify if the decrease in glycemia in this group is attributed to reduction of food intake, study #2 was carried out where controls were pair-fed with *V. vitis*-treated group. Both pair-fed and *V. vitis*-treated mice has similar loss in body weight, albeit pair-fed animals had a tendency to exhibit slightly greater cumulative weight loss (Figure 4; N.S., n=7 per group). *V. vitis* and pair feeding have significantly lower blood glucose levels (Figure 4; p<0.05, n=7 per group). This suggests that the effect of *V. vitis* could be mainly mediated through loss of appetite that induces reduction of food intake, body weight and gylcemia.

To test if *V. vitis* exerts the same effects in normal mice, study #3 was conducted in normal C57Bl/6J mice. In contrast to study#1 and study#2, no significant changes in body weight or glycemia were observed in *V. vitis*-treated animals as compared to vehicle controls (Figure 5; N.S., n=7 per group). Although, *V. vitis*-treated group of normal mice had a slightly higher tendency to lose weight than vehicle treated ones (Figure 5; N.S., n=7 per group), this group actually exhibited a slight, albeit significant, increase in cumulative food intake (Figure 5B; p<0,05, n=7 per group).

Cumulative fluid intake expressed was significantly reduced by *V. vitis* treatment in all the three studies as compared to their respective controls: by 44% in *V. vitis*-treated mice (study #1, Figure 3; p<0.05, n=7 per group), by 31% in pair-fed study (study #2, Figure 4; p<0.05, n=7 per group) and by 22% in normal mice (study# 3, Figure 5; p<0.05, n=7 per group).

Effects of *V. vitis* on insulinemia, triglyceridemia, and circulating leptin or adiponectin levels

V. vitis-treatment in KKA^y mice tended to reduce plasma insulin (by 46%) as compared to their control congeners (46 % decrease, Table 1; N.S., n=7 per group). In contrast, plasma triglyceride levels were decreased by 36% in the *V. vitis*-treated group, as compared to vehicle controls (Table 1, N.S., n=7 per group). However, because of data variability, these changes failed to reach statistical significance. Other blood lipid parameters including total Cholesterol, HDL-C and LDL-C, as well as plasma leptin and

adiponectin levels, and leptin/adiponectin ratio (Table 1) were not affected by *V. vitis* treatment.

Effects of *V. vitis* on liver steatosis, as well as liver and kidney functions

Liver lipid accumulation was assessed through histological analysis of hepatic tissue collected at sacrifice. In control KKA^y diabetic mice of study#1, only 1 out of 7 mice had grade 0 steatosis, in contrast to 3 out of 7 mice in *V. vitis*-treated group. On the other hand, 5 control animals had grade 3 steatosis, in contrast to only 3 animals in *V. vitis*-treated group (Table 2; $p<0.05$ by Chi square test, n=7 per group).

Interestingly, administration of *V. vitis* did not significantly affect liver or kidney function tests, albeit serum AST (liver), ALT (liver), creatinine (kidney) and LDH (muscle, kidney, and liver) levels in *V. vitis* treated diabetic mice all tended to be lower than corresponding values in control mice (Table 1).

Effect of *V. vitis* on GLUT4 content in skeletal muscle, phosphorylated ACC and PPAR-α protein content in liver of KKA^y mice

The skeletal muscle tissues of control, pair-fed and *V. vitis* treated mice were probed for their total content in GLUT4 protein. *V. vitis*- treatment tended to increase the total content of GLUT4 protein in soleus muscle compared to vehicle-treatment in study #1 and to pair-feeding in study #2 (Figure 6 A, B, N.S., n=7 per group).

To investigate whether the AMPK pathway is involved in the effects of *V. vitis in vivo*, we compared levels of phosphorylation of the downstream enzyme ACC in muscles and livers harvested from control, pair-fed and *V. vitis* treated diabetic KKA^y mice from the first two studies. Immunoblots showed that phospho-ACC content in the liver was similar in vehicle control and *V. vitis*-treated diabetic mice. In contrast, phospho-ACC content tended to be higher in *V. vitis* treated animals when compared to pair-fed animals in study #2 (Figure 6 C, E; N.S, n=7 per group). In addition, there was no significant difference in the phosphorylation levels of ACC in the muscles of control, pair-fed and *V. vitis*-treated mice (blots are not shown).

Consistent with decreased triglycerides content in the liver of *V. vitis*-treated animals, the content of PPAR- α in *V. vitis* treated groups in both studies tended to be

higher in comparison with control and pair-fed groups (Figure 6 C, D; N.S., n=7 per group). Due to small sample size, this difference did not reach statistical significance.

Discussion

Aboriginal populations around the world are particularly at risk of developing metabolic disorders related to lifestyle changes (Brassard et al., 1993), (Young et al., 2000),(Hegele, 2001). When risk factors like obesity and genetic predisposition entwine with the cultural disconnection of modern medication, the complications of diabetes can become debilitating and life threatening. To address this problem in Canadian aboriginal populations, our team has launched a project to explore potential antidiabetic plants stemming from Cree traditional medicine that could represent promising culturally relevant alternative and complementary treatment options for managing diabetes in these populations. In collaboration with the Cree Elders and healers of Eeyou Istchee (James Bay area of QC, Canada), we have identified the extract of the berries of *Vaccinium vitis-idaea* as a promising product that was capable of enhancing glucose uptake in skeletal muscle cells in culture (Harbilas et al., 2009). Subsequent bioassay guided fractionation studies on *V. vitis* resulted in the identification of quercetin and its glycosides, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside as the active principles responsible for the enhancement of glucose uptake in skeletal muscle cells (Eid et al., 2010).

Skeletal muscle cells express two isoforms of facilitative glucose transporters, GLUT4 and GLUT1. The later resides mainly in the plasma membrane and is responsible for basal glucose transport. On the other hand, GLUT4 resides in intracellular vesicles and translocates to the plasma membrane in response to insulin stimulation (Mitsumoto et al., 1991) (Giorgino et al., 2000). In accordance with previous observations (Harbilas et al., 2009), (Eid et al., 2010), *V. vitis* significantly increased cellular glucose uptake. The effect of *V. vitis* on glucose uptake in L6 cells (65% increase) was more than double of that observed previously in C2C12 myotubes (31%; (Eid et al., 2010)). This could be attributed to the higher content of GLUT4 in L6 cells (Sarabia et al., 1990), (Mitsumoto and Klip, 1992).

V. vitis-treatment also lead to the activation of insulin-independent AMPK and p38MAPK pathways but not of insulin-dependent Akt in L6 myotubes. This is in accordance with previous studies implicating the AMPK pathway and mitochondrial inhibition in the mechanism of action of *V. vitis* and its active principles (Eid et al., 2010). The use of selective AMPK inhibitors such as compound C deems necessary to prove the

involvement of this enzyme in stimulation of glucose uptake by *V. vitis* extract and will be the subject of future research.

This *V. vitis*-induced increase in glucose uptake could be the result of increased translocation of GLUT4 to plasma membrane. Therefore, the cell surface content of GLUT4 was measured in L6 cells expressing GLUT4 protein tagged with the *myc* epitopes. Results clearly confirm that *V. vitis* increases the translocation of GLUT4 to the plasma membrane of these cells. Interestingly, the extent of glucose transport stimulation and GLUT4 translocation induced by *V. vitis* administration was equivalent to that of an optimal dose of insulin in these cells.

Based on these and other recent promising *in vitro* results (Eid et al., 2010), as well as on the previously reported *in vivo* antidiabetic activity of quercetin and quercetin-3-*O*-glucoside (Kannappan and Anuradha, 2009), (Panda and Kar, 2007), (Shetty et al., 2004a), (Vessal et al., 2003), *V. vitis* was administered to diabetic KKA^y mice.

V. vitis extract administration exerted immediate, considerable and persistent effect to lower food intake. As a result of reduced food intake, cumulative change in body weight and glycemia were significantly reduced. These results suggest that the plant may possess important weight, blood-glucose and appetite-reducing effects. This led us to carry out study #2, utilizing pair feeding to determine whether the reduction of blood glucose concentration by *V. vitis* could be attributed to reduced food intake. This study revealed that pair-fed animals exhibited significantly lower levels of blood glucose than *V. vitis* treated animals, despite a fully comparable pattern of weight changes. One possible explanation for this counter-intuitive result stems from the significant sugar content of *V. vitis* berries that can reach 8% of their fresh weight (Hjalmasson and Ortiz, 2001) and about 27% of their dry weight. The sugar that *V. vitis* administration may thus provide actually enhances the significance of the anti-hyperglycemic and weight-reducing actions observed with plant extract treatment in diabetic KKA^y. Overall, these results therefore indicate that the antihyperglycemic effect of *V. vitis* is mediated, almost entirely, by the reduction of food intake. The underlying mechanisms remain to be elucidated, but they do not appear to involve the satiety hormone leptin whose circulating levels were not affected by *V. vitis*. Moreover, we found no evidence that the reduced food intake was related to any toxic action of the plant. Indeed, we did not observe any behavioral or external (e.g. fur, eyes)

changes indicating toxicity (not illustrated). In fact, several blood markers of toxicity (notably relating to liver and kidney function) actually tended to improve in *V. vitis* treated animals. Finally, the plant's berries have been and continue to be consumed by the CIE and should thus be considered GRAS (generally regarded as safe).

In contrast with KKA^y, the administration of *V. vitis* to normal C57Bl/6J did not affect body weight or blood glucose levels. A possible explanation to the difference in response to *V. vitis*-treatment in the two mouse strains might be linked to the disruption of central melanocortin (MC) system in KKA^y mice. Central MC system is a component of a circuit within the central nervous system (CNS) that regulates feeding behavior and energy expenditure. MC is modulated by a number of peripheral and central systems that regulate energy balance (Nogueiras et al., 2007).

The agouti (*A*) gene is normally expressed during the hair growth cycle in the neonatal skin where it functions as a paracrine regulator of pigmentation. KKA^y mouse model expresses dominant agouti alleles A^y, which results in ectopic expression of the agouti peptide in the ventromedial nucleus of the hypothalamus (VMH). In VMH, the agouti peptide antagonizes the melancortin receptor 4 (MC4-R), resulting in the KKA^y model phenotype of hyperphagia, hyperlipidemia, hyperleptinemia, maturity-onset obesity and diabetes (Nonogaki et al., 2006).

Similar in size and genomic structure to the agouti peptide, the agouti related peptide (AgRP) is a neuropeptide normally produced in the hypothalamus and the adrenal glands of humans and mice. It has been shown to be an antagonist of MC3-R and MC-4 which are directly linked to control of body weight (Einbond et al., 2004), (Ollmann and Barsh, 1999), (Havel et al., 2000). Therefore, the decrease in food intake observed in KKA^y mice during treatment with *V. vitis* could be the result of agonistic activity of *V. vitis* on MC-4R or antagonizing the agouti peptide in the hypothalamus. This makes *V. vitis* an interesting therapeutic agent in humans with genetic defects in or upstream of AgRP or MC-4R. Further research is needed to study the effect of *V. vitis* on melanocortin system especially that the biochemical and histological analysis indicate absence of toxicity.

In contrast with diabetic obese KKA^y mice, normal lean *V.vitis*- treated C57BL/6L mice had a slight but significant increase in food intake. This discrepancy of results may suggest that *V. vitis* treatment mimics the effect of chronic moderate to intense physical

activity. Regular moderate to vigorous exercise but not short light exercise performed by lean animals and humans, created a negative energy balance. This triggered a compensatory increase in food intake that did not affect body mass. Inversely, obese individuals did not change their food intake, probably due to their excess of energy storage (Mayer et al., 1954), (Woo et al., 1982b), (Woo et al., 1982a), (Melzer et al., 2005), (Slentz et al., 2004). In the same way, *V. vitis* could create a negative energy balance by increasing energy expenditure. This probably would be the result of disruption of mitochondrial energy transduction or to induction of uncoupling proteins (UCPs) as a consequence of increased mitochondrial biogenesis secondary to chronic activation of AMPK (Putman et al., 2003). Interestingly, it was previously reported that AMPK activation mimicked some of the metabolic changes associated with chronic exercise training (Putman et al., 2003).

V vitis-treatment had a remarkable ability to reduce water intake. Indeed, the plant's extract reduced blood glucose level by 32% whereas fluid intake dropped 3-fold. Several factors may have contributed to this effect. On average, KKA^y mice drank about 15 ml of water daily versus an average of 5.5 ml in normal C57BL/6 mice. Treatment of KKA^y mice with *V. vitis* for ten days normalized fluid consumption to levels closely similar to that of normal mice. Through its significant effect to reduce blood sugar, *V. vitis* should diminish blood tonicity and polyuria, two important determinants expected to increase fluid intake in KKA^y diabetic mice. Moreover, the reduction in the consumption of the dry chow diet, induced by *V. vitis*-treatment, could also be accompanied by a reduction in fluid intake. Finally, it is possible that the presence of 1 % of *V. vitis* extract in the drinking water of the mice have represented an unpleasant organoleptic feature, given that the plant's fruit, a close cousin of cranberries, shares its acidic and astringent properties. Another argument for the contribution of a taste related effect comes from study #2 carried out with pair-fed KKA^y mice. In this case, despite a reduction of blood glucose equivalent to that of *V. vitis* treated animals without consumption of the extract, pair-fed animals exhibited a much weaker drop in water intake, going roughly from 13 to 10 ml daily. Likewise, normal C57Bl/6 mice treated with *V. vitis* reduced water intake to 3.2 ml, from values of 5.6 in vehicle control normal mice, despite unchanged blood glucose concentrations. Hence, *V. vitis* likely reduces fluid intake by a combination of effects implicating reduced glycemia, diminished dry food intake and putative unpleasant organoleptic features.

Administration of *V. vitis* also tended to reduced liver steatosis in KKA^y and this may have contributed to the apparent improvement of insulin resistance. Indeed, hepatic steatosis is closely associated with obesity and insulin resistance, leading to exaggerated hepatic glucose production (Seppala-Lindroos et al., 2002). Peroxisome proliferator-activated receptor α (PPAR-α), a member of the nuclear hormone receptor superfamily, is essential for the regulation of hepatic fatty acid metabolism, as illustrated by the fatty liver that develops in PPAR-α knockout mice (Ip et al., 2003). In the present study, *V. vitis* administration tended to increased hepatic PPAR-α-content, consistent with improved liver steatosis.

Our *in vitro* results obtained in both C2C12 (Eid et al., 2010) and L6 muscle cells (herein) show that *V. vitis* increased the phosphorylation of AMPK and ACC. It is also known that the activation of the AMPK pathway in skeletal muscle can lead to increased synthesis of GLUT4 (Ojuka, 2004), which results in enhancing glucose transport capacity of skeletal muscle. The result of the present study thus show that *V. vitis* treatment *in vivo* tended to increases muscle GLUT4 content, thereby contributing to reduction in glycemia.

In summary, the results of the present study clearly demonstrate that *V. vitis* reduces food intake and body weight without inducing any toxicity. Moreover, the combination of *in vitro* and *in vivo* results presented herein suggests that the stimulation of the AMPK pathway leads to mobilization of GLUT4 transporters in skeletal muscle. These effects, combined with reduction of liver steatosis, significantly reduced glycemia and improved insulin sensitivity. The strong anti-obesity and antidiabetic potential of *V. vitis* warrants further clinical studies, notably in the context of a culturally relevant approach to diabetes care in the CEI.

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Figure Legends

Figure 1: *V. vitis* increased non-insulin-stimulated (basal) ^{3}H-deoxyglucose uptake and GLUT4 translocation in L6 GLUT4*myc* myotubes. Cells were treated with either 200 μg/ml of *V. vitis*, or with vehicle (0.1% DMSO) for 18 h. 100 nM insulin was applied for the last 20 min of the treatment in vehicle-treated cells. (A) Glucose uptake: Data are expressed after normalization to basal uptake of the vehicle control treated cells. Data are presented as the mean of 3 experiments ± SEM, each experiment composed of 3–4 replicates per condition. * Indicates a significant ($p \leq 0.05$) difference from the vehicle control group as assessed by ANOVA.

(B) GLUT4 translocation: Cell surface GLUT4*myc* was detected by an enzyme-linked colorimetric assay. Cells treated with *V. vitis* showed a 1.8-fold increase of GLUT4*myc* at the cell surface as compared to cells treated with vehicle control, whereas cells treated with insulin revealed a 1.75-fold increase as compare to vehicle treatment. Results represent the means ± SEM of three independent experiments, and 3-4 cells were analyzed for each condition per experiment. * Indicates a significant ($p \leq 0.05$) difference from the vehicle control group as assessed by ANOVA.

Figure 2: *V. vitis* increased phosphorylation of AMPK and p-38MAPK but not of Akt in L6 myotubes. Shown are representative immunoblots of cells treated for 18 h with either vehicle (0.1% DMSO) or 200 μg/ml of *V. vitis*. The lower blot was probed with anti-β-actin as a loading control. AICAR (1 mM) and insulin (100 nm) applied for the last 30 min of the treatment in vehicle-treated cells were used as positive controls for the activation of the AMPK pathway and for Akt phosphorylation, respectively.

Figure 3: *V. vitis* reduced cumulative change in body weight, cumulative change in food intake, non-fasting blood glucose concentration, and cumulative change in fluid intake in KKA^{y} mice of study #1. The study lasted for 10 days. *denotes significantly different as compared to control group ($p < 0.05$) as assessed by non paired t test.

Figure 4: Effect of 10 days *V. vitis*-treatment and pair-feeding on cumulative change in body weight, non-fasting blood glucose concentration, and cumulative change in fluid

intake in KKA^y mice of study #2. *denotes significantly different as compared to pair-fed mice ($p < 0.05$) as assessed by non-paired t test.

Figure 5: *V. vitis* has no effect on cumulative change in body weight, cumulative change in food intake, and non-fasting blood glucose concentration. It significantly reduced cumulative change in fluid intake in normal C57BL/6 mice of study #3. The study lasted for 10 days. All values are mean ± SEM (n=7), *denotes significantly different as compared to control mice ($p < 0.05$) as assessed by non-paired t test.

Figure 6: Effect of *V. vitis* on GLUT4 levels in skeletal muscles, phosphorylation of ACC and PPAR-α levels in livers from diabetic KKA^y mice of study #1 (A) and study #2 (C). Samples of soleus muscles and liver tissues were obtained from control, *V. vitis* and pair-fed diabetic KKA^y mice and analysed by immunoblotting with antibodies specific to phospho-ACC, Glut4 and PPAR- α. Quantification of GLUT4/β-actin (B), quantification of PPARα/β-Actin (D), and quantification of p-ACC/ β-actin (E). Immunoblots are representative of results obtained from seven animals in each group.

Table 1 Blood parameters of KKA^y mice from study #1

	Control	***V. vitis***
AST (IU/L)	107.7 ± 14.5	92.0 ± 11.7
ALT (IU/L)	64.5 ± 8.7	56.0 ± 10.4
LDH (IU/L)	108.8 ± 9.4	71.5 ± 18.0
Creatinine (IU/L)	1280.7 ± 340.2	832.7 ± 219.9
Alkaline phosphatase (IU/L)	112.4 ± 11.1	77.0 ± 7.9*
Triglycerides (mmol/L)	4.5 ± 0.8	2.9 ± 0.5
Cholesterol (mmol/L)	2.3 ± 0.14	2.12 ± 0.19
HDL (mmol/L)	1.07 ± 0.09	1.09 ± 0.11
LDL (mmol/L)	0.5 ± 0.2	0.5 ± 0.0
Insulin (ng/ml)	35.32 ± 8.48	18.98 ± 4.22
Leptin (ng/ml)	27.12 ± 1.7	23.9 ± 0.99
Adiponectin (µg/ml)	18.17 ± 2.44	18.00 ± 2.2

Measurements were obtained from plasma of mice treated for a period of 10 days with standard Chow diet with or without *V. vitis* in the drinking water (1 g%). All values are mean ± SEM (n=7). * denotes significantly different as compared to Control ($p < 0.05$) as assessed by non-paired t test.

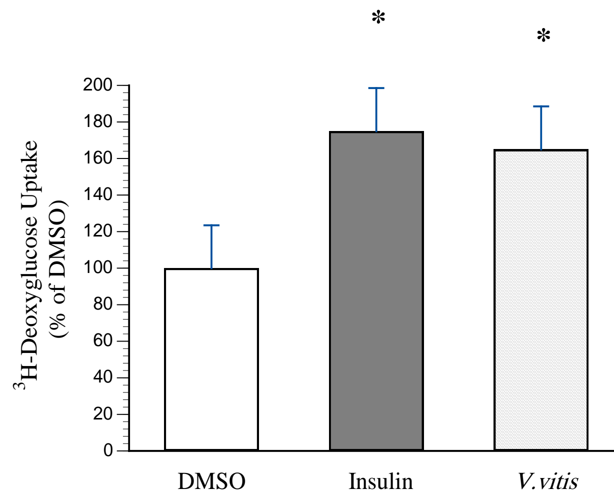
Table 2 Histological scores of liver steatosis from control and *V. vitis* treated KKA^y diabetic mice.

Groups	***n***	**Steatosis**			
		0	**1**	**2**	**3**
Control	7	1	1	0	5
V .vitis	7	3	1	0	3

The scoring is based on the percentage of hepatocytes containing macrovesicular steatosis, grade 0: 0-5 %, grade 1: 5-33 %, grade 2: 33-66 %, grade 3: more than 66 % (Brunt et al., 1999), (Kleiner et al., 2005).

Figure 1

A



B

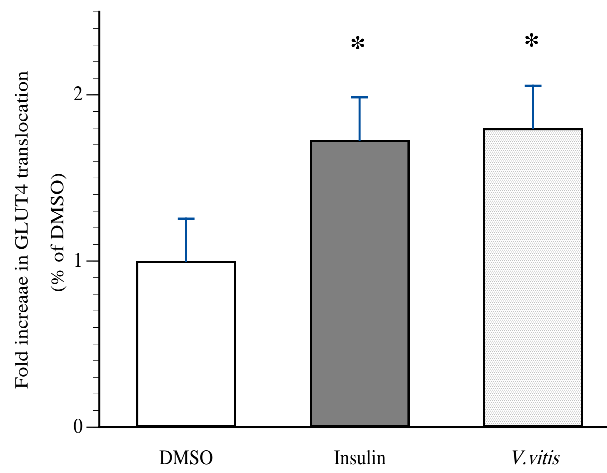


Figure 2

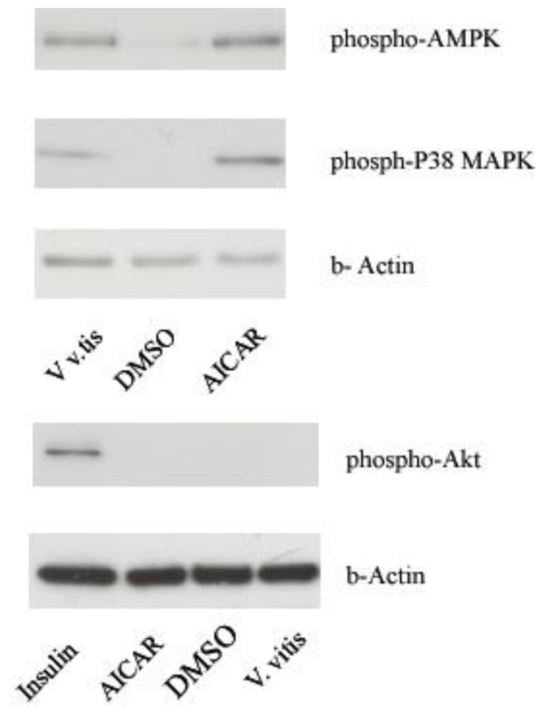
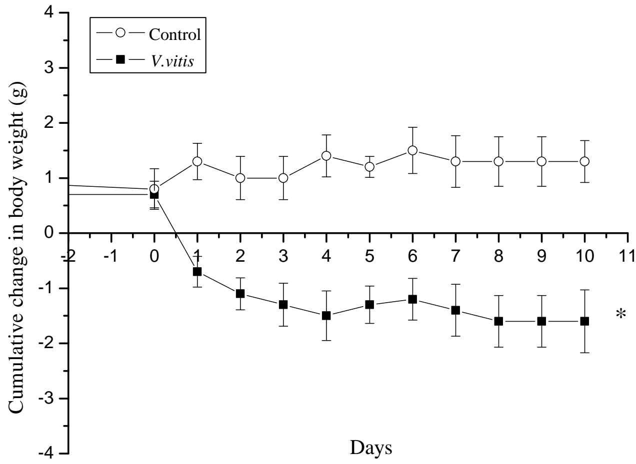
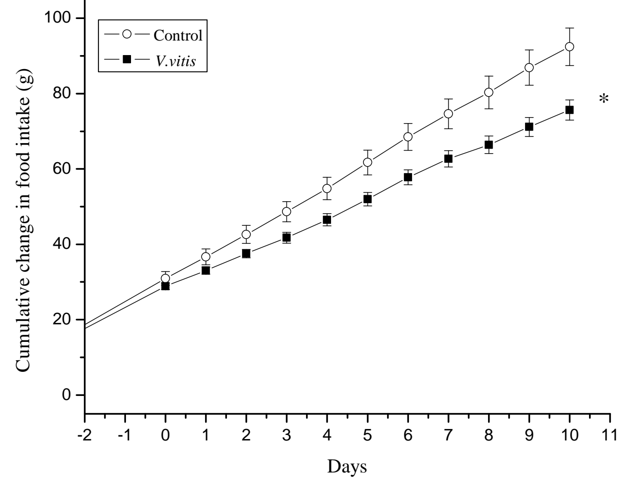
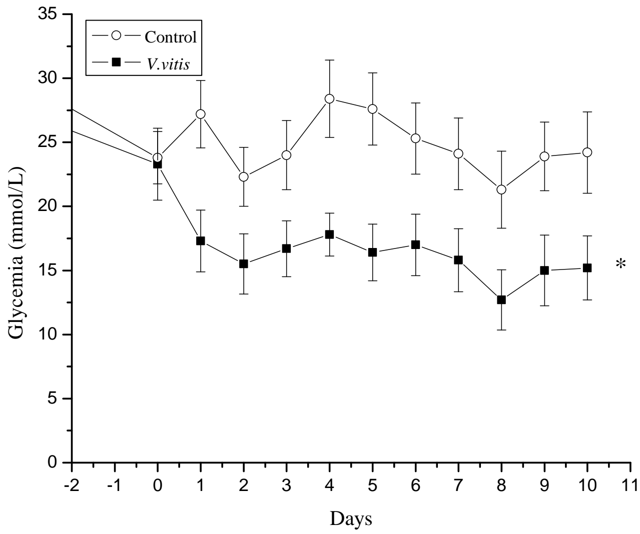


Figure 3

Study#1 KKA^y mice







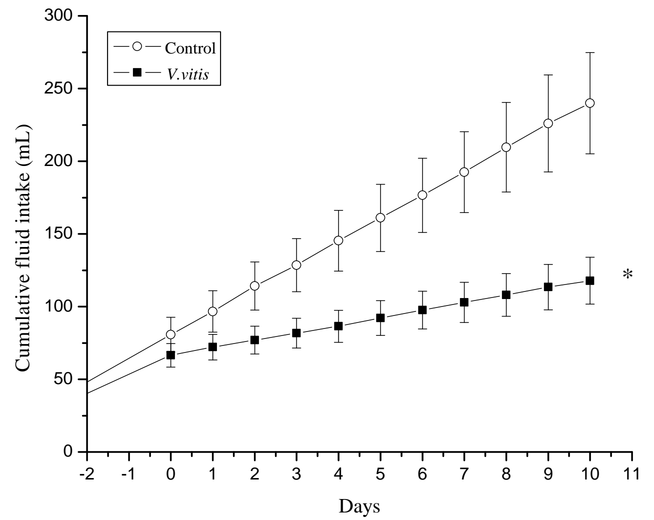


Figure 4

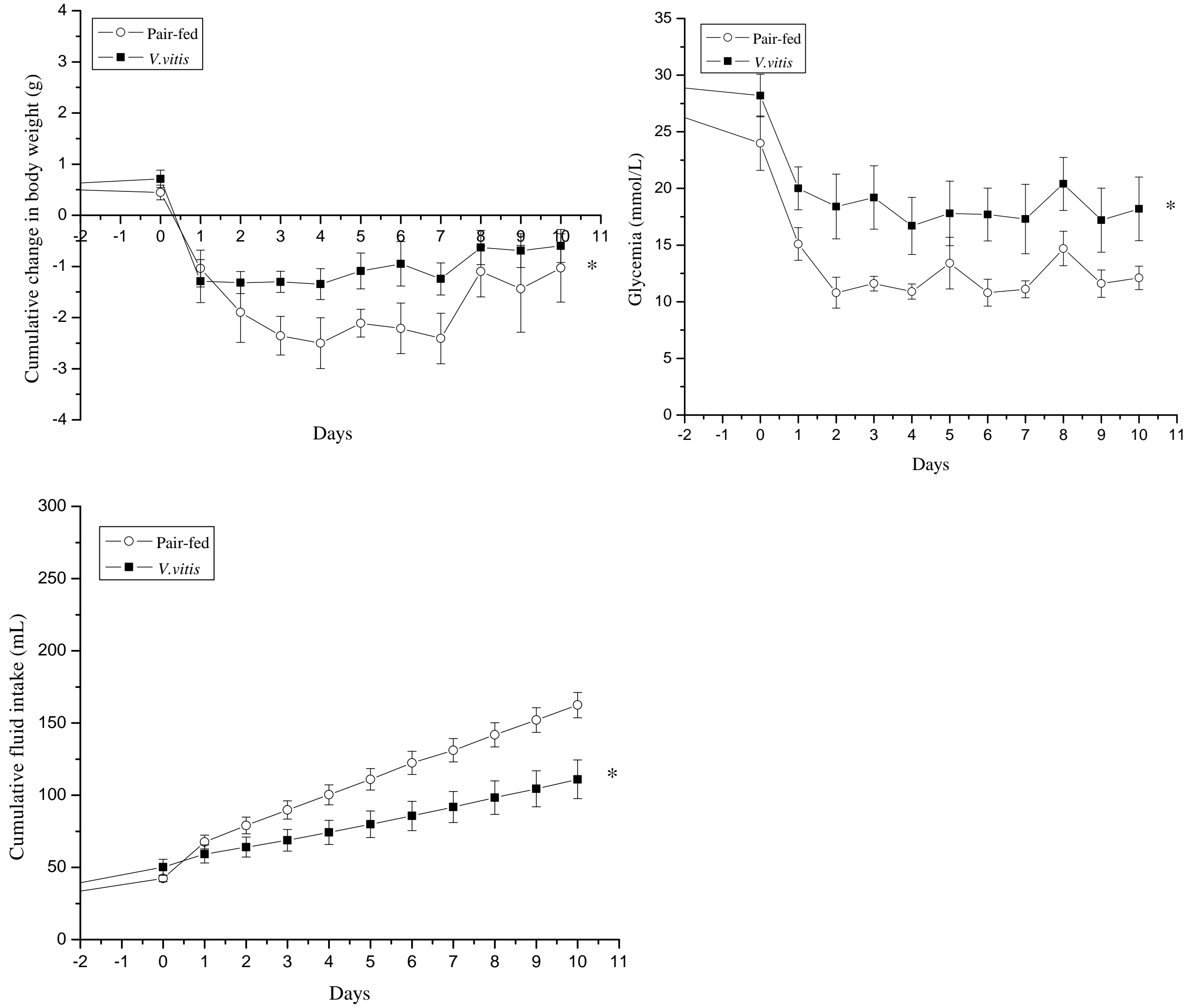


Figure 5

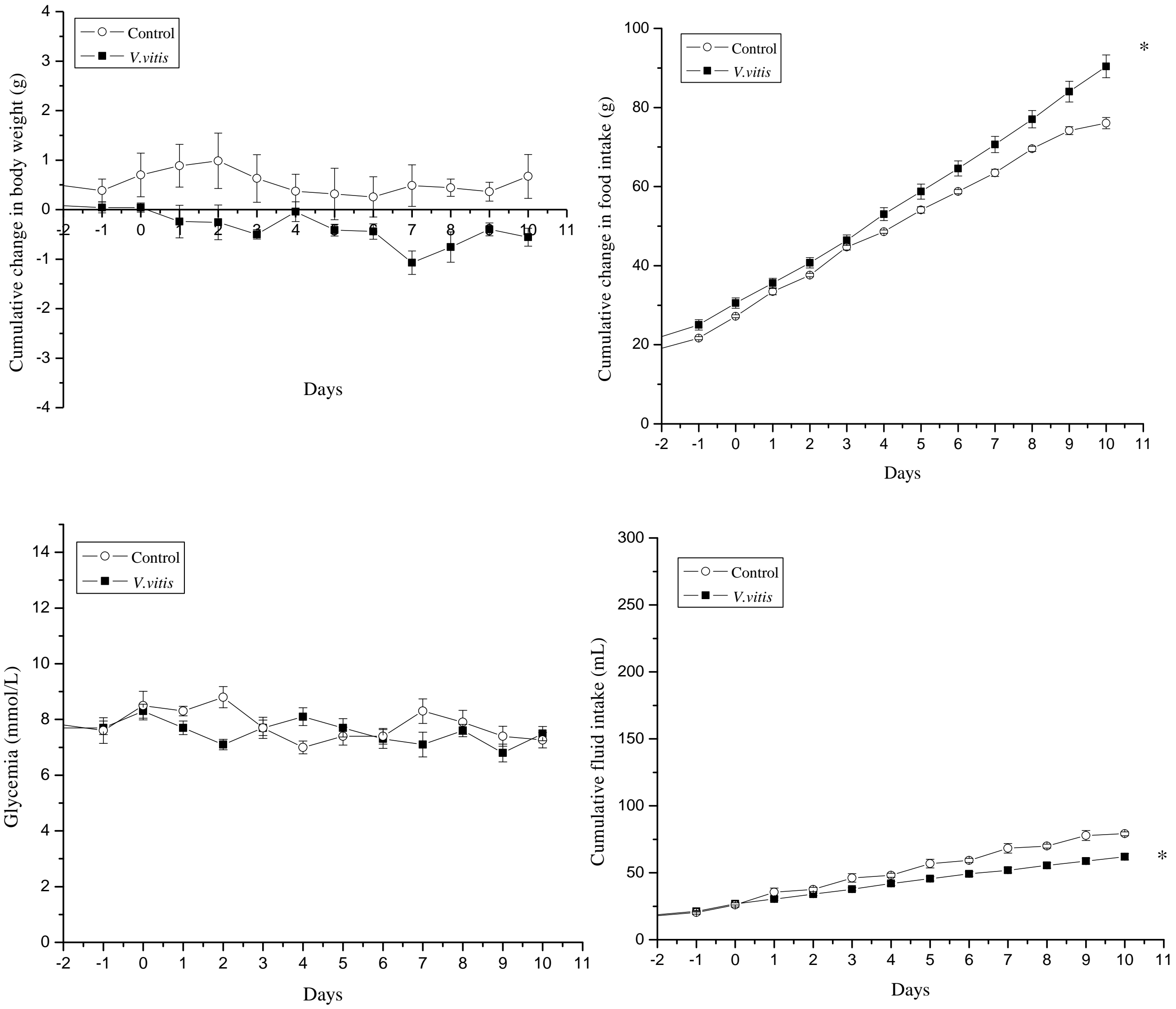
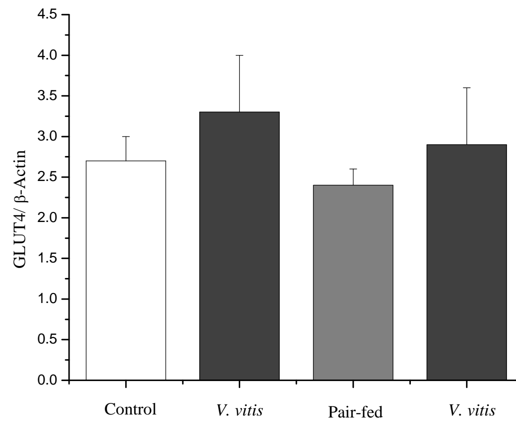


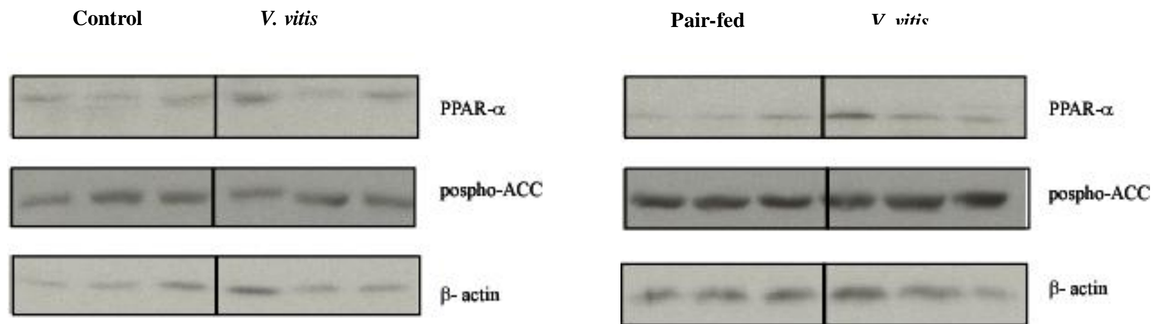
Figure 6

A

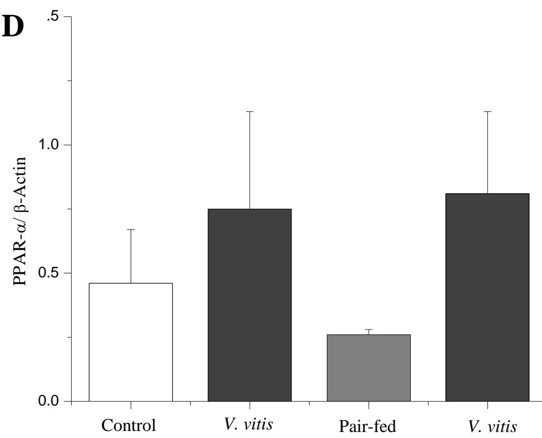
B



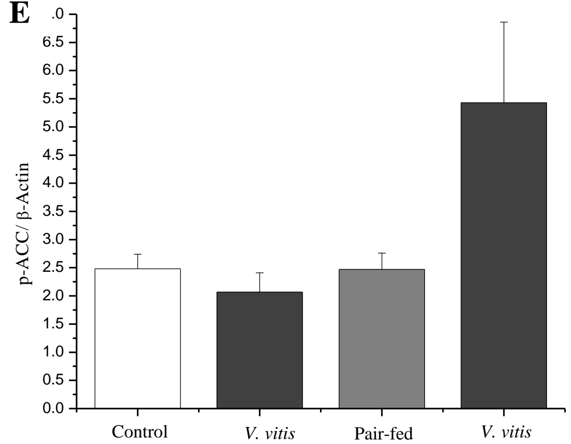
C



D



E



5. General discussion

Due to the growing western interest in herbal medicine, and the increasing need for effective treatment for many diseases, medicinal plants have been the focus of intense research by industry and academia to validate the efficacy of traditional medicine and to assess their safety (Taylor et al., 2001). With many clinical trials being conducted and published every year, evidence-based herbal medicine continues to gain acceptance from health care providers. Nowadays, many international organizations and governmental agencies support projects and programs in this area (Sackett et al., 1996), (Yeh et al., 2003). Research into the scientific validation of Cree traditional plants used to treat symptoms of diabetes is the current focus of our project sponsored by the Canadian Institutes of Health Research (CIHR).

In Canada, diabetes was unheard of in the Aboriginal populations in the first half of the 20th century (Hegele, 2001). Over the few past decades, the age-adjusted rate of type 2 diabetes in Aboriginal peoples has greatly increased to become 3 to 5 times higher than that in the general population. Due to the disproportionate high rates of complications, and the development of type 2 diabetes in young children and adolescent, diabetes is now considered the leading cause of morbidity and mortality in aboriginal communities throughout Canada (Brassard et al., 1993),(Young et al., 2000), (Hegele, 2001).

The holistic nature of traditional aboriginal medicine makes it different from conventional western medicine. While western medicine focuses on the diagnosis and treatment of the disease with unconditional belief in science as the unique source for knowledge, traditional medicine through recognizing the social, emotional, spiritual and physical dimensions of wellness, is more concerned with the whole person rather than the symptoms of the disease itself (van Uchelen et al., 1997). This gap between the two approaches has created a condition of poor compliance of aboriginals to western medical advice and has called attention to the urgent need for culturally appropriate community-based prevention and treatment programs to help slow down the diabetes epidemic (Macaulay and Ryan, 2003). In this context, it is important to note that traditional medicine is an integral part of Aboriginal culture, through its recognition and integration in the health

care system, the delivery of health services to Aboriginal communities can be markedly improved (Devanesen and Maher, 2003).

In an effort to address this cultural disconnection of diabetes care, our team has been working on identifying safe and efficacious alternative treatments for diabetes based on these populations' traditional medicine and pharmacopeia. In collaboration with the CEI (Eastern James Bay Cree), we have identified the medicinal plants used to treat symptoms of diabetes in four CIE communities. Two screening studies conducted in our laboratories revealed that eight products enhanced glucose uptake in skeletal muscle cells (Spoor et al., 2006), (Harbilas et al., 2009). In one of these screening studies, *V. vitis* was as potent as the biguanide oral anti-hyperglycemic drug metformin in promoting glucose uptake in C2C12 myotubes (Harbilas et al., 2009).

Generally speaking, antidiabetic plants, whose molecular and cellular targets of action have been investigated, fall into one or more of the four principal categories: insulin sensitizers, insulin mimetics, insulin secretagogues and inhibitors of intestinal carbohydrate digestion and absorption.

Targeting mitochondrial energy transduction via inhibition of complex I of the respiratory electron transport chain and subsequent AMPK activation mediate the effect of the two popular insulin sensitizers biguanides and thiazolidinediones (Elia et al., 2006), (Brunmair et al., 2004). Interestingly, 7 plant products from the pharmacopea of the CEI as well as several naturally occurring compounds, including many flavonoids, exert their antidiabetic activity via either inhibition or uncoupling of mitochondrial oxidative phosphorylation (Martineau et al., 2009), (Polya, 2003). Similarly, the juice of *Vaccinium angustifolium* (blueberry native to eastern and central Canada), biotransformed with *Serratia vaccinii*, a new strain of bacteria isolated from blueberry fruits, was also reported to activate AMPK (Vuong et al., 2007).

Insulin sensitizers also include products that regulate hepatic glucose and glycogen metabolism. Plants belong to this category include garlic (*Allium sativum*) and onion (*Allium cepa*), which decrease blood glucose levels by normalizing liver hexokinase and G-6-Pase activities (Sheela et al., 1995). Similarly, *Coccinia indica* and *Momordica charantia*

were reported to normalize the activity of G-6-Pase (Singh et al., 1989), (Shibib et al., 1993). Moreover, *C. indica* pectin stimulated glycogen synthetase and suppressed glycogen phosphorylase and hepatic fructose-1,6-bisphosphatase in normal rats (Kumar et al., 1993), (Shibib et al., 1993).

Nigella sativa and *Cinnamomon cassia* (cinnamon) were suggested to have insulin mimetic properties, through enhancing insulin signaling pathway independently of insulin (Benhaddou-Andaloussi et al., 2008) (Qin et al., 2003).

Gymnema sylvestre, *N. sativa and M. charantia* exert part of their anti-hyperglycemic effect by inhibiting glucose absorption in the small intestine (Meddah et al., 2009), (Persaud et al., 1999), (Matsuda et al., 1998). On the other hand, alkaloids of *Morus alba* (Asano et al., 1994), as well as the aqueous and ethyl acetate soluble fractions of *Salacia oblonga* inhibit glucose digestion by inhibiting α-glucosidase activity (Matsuda et al., 1998).

Stimulation of insulin secretion constitute the most reported mechanism of action of many antidiabetic plants including cinnamon (Khan et al., 2003), *N. sativa* (Benhaddou-Andaloussi et al., 2010), *Acacia arabica* (Singh et al., 1975), (Wadood et al., 1989), *Aloe vera* (Ajabnoor, 1990), *Citrullus colocynthis* (Abdel-Hassan et al., 2000), *G. sylvestre* (Asano et al., 1994), *Morus alba* (white mulberry) (Asano et al., 1994), (Singh et al., 1989), and *Trigonella foenumgraecum* (Fenugreek) (Vats et al., 2003).

In a previous study, *V. vitis* were reported to exert no effect on glucose stimulated insulin secretion by pancreatic β-cells in culture (βTC) (Harbilas et al., 2009). Moreover, in contrast with the strong *in vitro* inhibitory effect of on intestinal glucose uptake *V. vitis,* the extract failed to inhibit glucose absorption by the intestine of normal Wistar rat subjected to OGTT (Nistor et al., under publication).

The findings of the present thesis show that *V .vitis* is an insulin sensitizer. Indeed, *V. vitis* increased glucose uptake in murine C2C12 and rat L6 muscle cells. This effect is the result of at least in part to activation of AMPK following the anabolic stress induced by inhibition of mitochondrial respiration. The use of selective AMPK inhibitors such as compound C deems necessary to prove the involvement of this enzyme in stimulation of

glucose uptake by *V. vitis* extract and will be the subject for future work. Rat L6 cells transfected with GLUT4*myc* displayed membrane localization upon treatment with this plant, indicating GLUT4 implication in *V. vitis* action. As with other species tested in a previous study (Spoor et al., 2006), *V. vitis* berry extract did not stimulate Akt phosphorylation suggesting that the insulin-signaling pathway is not involved in the mode of action of *V. vitis* in both cell lines.

Identification of active compounds in medicinal plants allows the comparison of traditional preparations between communities during different seasons thus providing better quality control over geographical and temporal variations. For this purpose, fractionation of *V. vitis* berry extract guided by muscle cell glucose uptake was carried out and resulted in the isolation of quercetin and quercetin-3-*O*-glycosides as the main active principles responsible for the enhancement of glucose uptake. These compounds were found to stimulate phosphorylation of AMPK and ACC. In addition, *V. vitis* caused a mild inhibition of mitochondrial respiration, while quercetin was found to be a potent inhibitor. Such an inhibitory effect of quercetin was previously reported and was attributed to direct binding of quercetin to the F1-ATPase (Gledhill et al., 2007). Therefore, the same mechanism of action seems to be at the base of the antidiabetic activity of both the crude extract and its active compounds. Metabolic acidosis is a common side effect of powerful disruptors of oxidative phosphorylation and ATP lowering agents and was behind the withdrawal of drugs such as phenformin from the pharmaceutical marketplace. Interestingly, quercetin inhibition of mitochondrial respiration did not result in the increase of extracellular acidification rate or decreased cellular content of ATP. This metabolic and safety profile makes *V. vitis* and its active compound quercetin attractive antidiabetic agents.

A derivative of caffeic acid, caffeic acid methyl ester (CAME), was also isolated during the biologically guided fractionation of *V. vitis*. This compound was later understood to be a by-product formed as a result of using methanol as the solvent during the fractionation process. Among *V. vitis* sub-fractions and pure compounds tested for enhancing glucose uptake, CAME had the most pronounced effect in glucose uptake assays performed in C2C12. It was previously reported that another derivative of caffeic acid,

caffeic acid phenethyl ester (CAPE), exhibited anti-diabetic activity in the form of potent AMPK-mediated stimulation of glucose uptake in skeletal muscle cells (Lee et al., 2007).

Our study attempted to elucidate the mechanism by which CAME and CAPE exert their anti-diabetic activity and to identify other caffeic acid derivatives with similar activity. We also evaluated the relationship between the activity and the cytotoxicity of these compounds on the one hand, and their molecular structure and physicochemical properties on the other hand.

Along with CAME and CAPE, other closely related caffeic acid esters were tested for glucose uptake stimulating activity as well as mitochondrial uncoupling activity. These included caffeic acid ethyl ester (CAEE), caffeic acid diallyl ester (CAAE), and caffeic acid n-octyl ester (CAOE). All the tested compounds potently increased basal uptake through activation of AMPK in response to an uncoupling effect on oxidative phosphorylation, a mechanism analogous to that of the classical uncoupler 2,4-dinitrophenol (Bashan et al., 1993). It was also reported that the disruption of ATP production in L6 skeletal muscle cells by DNP causes the efflux of calcium from the mitochondria. Calcium chelation and inhibitors of PKC inhibited DNP-mediated glucose uptake but not AMPK activation. It was thus concluded that calcium and cPKC partly mediate the stimulation of glucose uptake in L6 skeletal muscle cells by DNP (Khayat et al., 1998; Patel et al., 2001). The possibility that calcium plays a role in the increase of glucose uptake by caffeic acid esters requires further testing.

As is the case with the biguanides, mitochondrial uncouplers can potentially cause lactic acidosis. Therefore, the safety of caffeic acid derivatives can only be maximized at the cost of activity. Sixteen other related compounds were selected to address specific structure–activity hypotheses. Of these, none exhibited uncoupling activity nor significantly stimulated muscle cell glucose.

The results demonstrated that an intact caffeic acid moiety, devoid of strongly ionisable groups is essential for activity. Lipophilicity was also found to be a strong predictor not only of both uncoupling and glucose uptake stimulating activities but also of toxicity expressed as reduced cell viability. The best compromise, in this case, is CAME

which still exhibits an interesting increase in glucose uptake (65% at 50 μM), without negatively affecting cell viability. Due to its outstanding activity to cytotoxicity ratio, CAME appears promising and seems worthy of further studies investigating the potential of this compound and of other related derivatives as antidiabetic products.

Finally, the antidiabetic activity of *V. vitis* was validated in a type 2 diabetic animal model, namely KKA^y mice. The results indicated that, despite the possibility of providing slight extra calories due to its sugar content (Hjalmasson and Ortiz, 2001), *V. vitis* exhibited potent and immediate antihyperglycemic, appetite- and weight-reducing effects. Indeed, the pair-feeding study suggested that the antihyperglycemic and antiobesity effects of *V. vitis* were mediated, to a great extent, by the reduction of food intake. Other effects such as improvement in insulin sensitivity, a tendency towards reduction of plasma and liver triglycerides, activation of AMPK, stronger expression of PPAR-α in the liver and a tendency to increase GLUT4 content in the muscle also contributed to the antidiabetic activity of *V. vitis* extract.

On the other hand, the administration of *V. vitis* to normal C57BL/6J neither altered blood glucose levels nor reduced body weight, which suggests an activity of *V. vitis* on the defective melancortin system in KKA^y brain. Thus *V. vitis* could be an interesting therapeutic agent in humans with eating disorders related to central melancortin system. Further research is needed especially that the biochemical and histological analysis indicate absence of toxicity.

The overall results of the *in vivo* study make *V. vitis* a valuable therapeutic agent in a culturally adapted approach to diabetes care of Cree communities who are not ready to give up their traditional preparations for modern pharmaceuticals dosage forms. By identifying the active principles, the target sites and the mode of action, this thesis provides tools for phytochemical analysis, standardization and quality control of the traditional preparations. A process that is necessary to ensure efficacy, safety and consistency in composition and biological activity of traditional preparations.

6. Conclusion and perspective

The results of this thesis demonstrate that *V. vitis* berries extract possesses promising anti-hyperglycemic activities. Indeed, *in vitro*, *V. vitis* stimulated glucose uptake in murine C2C12 and rat L6 muscle cells in culture by promoting GLUT4 translocation to the membrane. These effects are thought to be downstreams of mitochondrial respiration inhibition and AMPK pathway activation. Interestingly, our *in vivo* studies showed that *V. vitis* exhibited significant anti-hyperglycemic and anti-obesity effects in diabetic KKA^y mice due to appetite reducing properties on one hand. On the other hand, *V. vitis* tended to increase the content of GLUT4 in KKA^y muscle and to reduce hepatic triglyceride content (probably due to increased PPAR-α protein levels). Our study suggests that *V. vitis* has a potential clinical utility in treating diabetes. However, detailed understanding of the mode of action of *V. vitis* will be necessary for its appropriate use. Several key factors remain to be considered.

As discussed earlier, treatment of type 2 diabetes revolves around controling circulating glucose levels (either through glucose production or utilization or through increasing insulin secretion and effectiveness) or by reduction of energy intake or increasing energy expenditure. Therefore, in addition to the mechanisms of glycemic control observed in this thesis, *V. vitis* effect could stem from the effect of this plant extract on other glucose transporters. We observed increased glucose uptake by C2C12 myotubes, although they do not sufficiently express GLUT4. GLUT1, hence might account for a significant portion of glucose transport in this skeletal muscle cell line (Nedachi and Kanzaki, 2006). In addition, the increase in GLUT1 mediated glucose uptake is reported to be associated with activation of AMPK (Barnes et al., 2002). It is thus conceivable that modulation of GLUT1 translocation and expression can be a mechanism that contributes to the antihyperglycemic effect of *V. vitis* in skeletal muscle and need to be investigated.

Secondarily, it is well established that the liver plays an important role in promoting glucose homeostasis. Indeed, in type 2 diabetes, hepatic glucose production is the main cause of fasting and postprandial hyperglycemia (Postic et al., 2004). The liver was also reported to be the target of antidiabetic drugs such as metformin and TZDs. These two

drugs suppress hepatic glucose production by activating hepatic AMPK. Thereby, another potential mechanism of *V. vitis* would be the activation of AMPK in hepatic cells and the subsequent reduction of hepatic glucose output mediated by inhibition of gluconeogenic enzymes (such as G-6-Pase, PEPCK and fructose-1,6-biphsophate). Further studies are needed to assess this potential effect of *V. vitis* on the liver and its control of glucose homeostasis.

Thirdly, reduction of energy intake is a very important strategy in diabetes and obesity management. The satiety-inducing effect of *V. vitis* could be attributed to inhibition of AMPK in the brain. Recent studies have shown that activation of hypothalamic AMPK increased food intake to restore energy balance (Kim and Lee, 2005). In contrast to activation of AMPK in liver and skeletal muscle, metformin inhibits AMPK in the hypothalamus. This mechanism was proposed to be at the base of the anorexic effect of metformin (Han et al., 2005). Furthermore, appetite regulating hormones like leptin and ghrelin were reported to regulate hypothalamic AMPK (Gao et al., 2007b). Therefore, it would be interesting to investigate the effect of *V. vitis* on hypothalamic AMPK. If *V. vitis* inhibits hypothalamic AMPK, this might explain the decrease in appetite observed upon treatment with *V. vitis*. Another possible target of *V. vitis* in the hypothalamus is the AgRP. Neuropeptide Y (NPY) is another orexigenic peptide that is co-expressed with AgRP in the arcuate nucleus of the hypothalamus. Hence, it would be important to study the effect of *V. vitis* on the expression of these two neuropeptides and their regulation of the melanocortin system which is responsible for feeding behavior.

Lastly, increasing energy expenditure is an important target in weight management strategies and control of diabetes. Mitochondria play a fundamental role, not only in energy production, but also in energy dissipation through thermogenesis. Uncoupling proteins (UCPs) are mitochondrial proteins involved in energy expenditure. UCP-1 is present exclusively in brown adipose tissue in rodents and is responsible for thermogenesis during cold exposure. Recent research has provided evidence that brown adipose tissue is also found in the upper part of the body of adult human (Nedergaard et al., 2007). On the other hand, UCP-2 is found in white fat, while UCP-3 is highly skeletal muscle specific (Nordfors et al., 1998), (Zhou et al., 2000). It is worthy to note that expression of UCP-3 is

upregulated by chronic AMPK activation (Putman et al., 2003). Therefore, mediated by activation of AMPK, *V. vitis* could increase the expression of these thermogenic proteins. Indeed, upregulation of UCP-3 is a part of the response to enhanced mitochondrial biogenesis (Zhou et al., 2000). The co-transcription factor, peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α) is an essential element in mitochondrial biogenesis and was shown to be regulated by factors such as activation of AMPK and calcium/calmodulin-dependent protein kinase IV (Atherton et al., 2005), (Liu et al., 2009). Recent studies showed that oxidative damage leading to decreased mitochondrial number and function is associated with a wide variety of diseases including insulin resistance, obesity and diabetes. Therefore, we hypothesize that *V. vitis* enhanced expression of PGC-1α, UCP-3 and increased mitochondrial biogenesis as a major response to activation of AMPK in muscle (Liu et al., 2009), (McCarty et al., 2009).

Our thesis has also shown that caffeic acid methyl ester (CAME) holds great potential for development as antidiabetic agents. Future work will be devoted to enable better understanding of the mechanism of the antidiabetic activity and the molecular targets of CAME and CAAE and to validate their antidiabetic properties in mouse model of type 2 diabetes.

Notwithstanding all the work still necessary, the present thesis represents a major contribution to the validation and understanding of the antidiabetic potential of *V. vitis*.

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