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Effets de plantes réputées antidiabétiques sur un modèle cellulaire hépatique de résistance à l'insuline induite par le palmitate

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Résumé

La pharmacopée Cris est riche en plantes médicinales et plusieurs d'entre elles sont étudiées par notre laboratoire pour leur potentiel antidiabétique. Certaines espèces ont démontré leur capacité à stimuler la protéine kinase activée par l'AMP (AMPK), une enzyme qui favorise la translocation de transporteurs de glucose à la membrane (effet hypoglycémiant). L'AMPK stimule également d'autres fonctions, telle l'oxydation des graisses, dans le but de rétablir l'énergie cellulaire. Ce projet a comme objectifs d'évaluer, premièrement, le stress métabolique induit par huit des extraits dans des cellules musculaires et des hépatocytes, effet qui serait responsable de l'activation de l'AMPK. Ce stress peut être déterminé en mesurant l'acidification du milieu extracellulaire ainsi que la déplétion du contenu en ATP des cellules suite aux traitements. Le deuxième objectif est de mesurer l'efficacité des extraits à réduire le contenu en gras (oxydation des graisses) et à ainsi normaliser la résistance à l'insuline dans des hépatocytes rendus insulino-résistants. Les hépatocytes sont rendus résistants à l'insuline (condition fortement lié à l'obésité) via traitement avec un acide gras saturé, le palmitate. Les résultats montrent que la majorité des extraits semble induire un stress métabolique de courte durée dans les cellules. Parmi les extraits, seul un a réussi à faire diminuer significativement le taux de triglycérides intracellulaire suite au traitement au palmitate sans toutefois améliorer la sensibilité à l'insuline. En conclusion, le potentiel hypoglycémiant des extraits serait du à leur capacité à affecter la respiration mitochondriale (stress métabolique). Toutefois, leur capacité à améliorer la sensibilité à l'insuline n'a pu être établie.

Mots-clés : Diabète de type II, populations autochtones, plantes médicinales, AMPK, stress métabolique, obésité, palmitate, résistance à l'insuline, oxydation des graisses

Abstract

Cree pharmacopeia is rich in medicinal plants and many of them are studied by our laboratory for their antidiabetic potential. Some of the species tested have shown to activate the AMP-activated protein kinase (AMPK), an enzyme responsible for the translocation of glucose transporters to the cell membrane (hypoglycaemic activity). AMPK is also known to activate other cellular functions, like fat oxidation, in order to restore cell energy loss. The objectives of this study are, first, to measure the metabolic stress induced by eight of the species in muscular and liver cells, an effect believed to be responsible for the AMPK activation. Metabolic stress is evaluated by measuring extracellular medium acidification and cellular ATP depletion. The second objective is to assess the capacity of the extracts to clear intracellular fat (fat oxidation) and, by doing this, restore insulin sensitivity in insulin-resistant driven hepatocytes. To become insulin-resistant (a condition strongly linked to obesity), the hepatocytes are treated with a saturated fatty acid, palmitate. The results show that most of the extracts seem to increase the metabolic stress in muscular cells and hepatocytes for a short period of time. Among all extracts, only one has significantly reduced intracellular triglycerides in palmitate treated hepatocytes, an effect not followed by an increase in insulin sensitivity. In conclusion, the species tested in this study seem to exert their hypoglycaemic potential by affecting mitochondrial respiration (metabolic stress). However, the experimentations have not clearly shown the capacity of the species to restore insulin sensitivity in insulin-resistant liver cells.

Keywords: Type II diabetes, aboriginal populations, medicinal plants, AMPK, metabolic stress, obesity, palmitate, insulin resistance, fat oxidation

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1. Introduction

1.1. Le diabète

Le diabète est une maladie métabolique qui se caractérise par une augmentation chronique de la glycémie (taux de glucose dans le sang). Il existe principalement deux types de diabète; le diabète de type I et le diabète de type II. Le diabète de type I (5 à 10% de tous les cas de diabète), ou insulinodépendant, résulte d'un dysfonctionnement dans la production et la sécrétion d'insuline par les cellules β des îlots de Langerhans du pancréas. Ce dysfonctionnement serait principalement dû à la destruction des cellules β par le système immunitaire (maladie auto-immune) et apparaît dans la majorité des cas à l'enfance (WHO, 1999). Le diabète de type II, ou non-insulino dépendant, résulte lui d'une déficience dans l'action de l'insuline au niveau de ses récepteurs (résistance à l'insuline) qui mène à une déficience relative dans la production et la sécrétion d'insuline. C'est la principale forme de diabète (85% des cas de diabète), qui apparaît le plus souvent à l'âge adulte et chez les personnes en surpoids (WHO, 1999). Les causes du développement du diabète de type II sont, en plus de prédispositions génétiques, les mauvaises habitudes alimentaires (consommation excessive d'aliments gras et sucrés), le manque d'activité physique et l'obésité (Hu et al, 2001).

Si non-traitée, la perte de contrôle de la glycémie induite par le diabète peut avoir des conséquences graves sur la santé de l'individu qui en est atteint. L'hyperglycémie affecte les petits vaisseaux au niveau de la rétine, des reins et des nerfs périphériques. Ceci peut amener, à la longue, au développement de la cécité, à une insuffisance rénale chronique ou encore à l'ulcération des extrémités (Fioretto, 2009). Un haut taux de glucose au niveau des gros vaisseaux est, quant à lui, un facteur de risque majeur

d'infarctus du myocarde et d'accidents cérébrovasculaires (Lehto et al, 1996; Haffner et al, 1998).

Le diabète est connu depuis l'antiquité dans de nombreuses cultures (égyptienne, indienne, chinoise, grecque, …), mais il connait depuis quelques décennies une augmentation fulgurante, principalement en ce qui concerne le diabète de type II (Amos et al, 1997). On estimait qu'en 2000, près de 150 millions d'individus étaient atteints du diabète à travers le monde et que ce chiffre devrait dépasser 300 millions d'individus diabétiques en 2025 (Wild et al, 2004). Le diabète touche principalement les pays industrialisés, mais le phénomène commence également à prendre des dimensions épidémiques dans les pays en développement car, les gens adoptent de plus en plus un style de vie et une alimentation de type « occidental » (Wild et al, 2004).

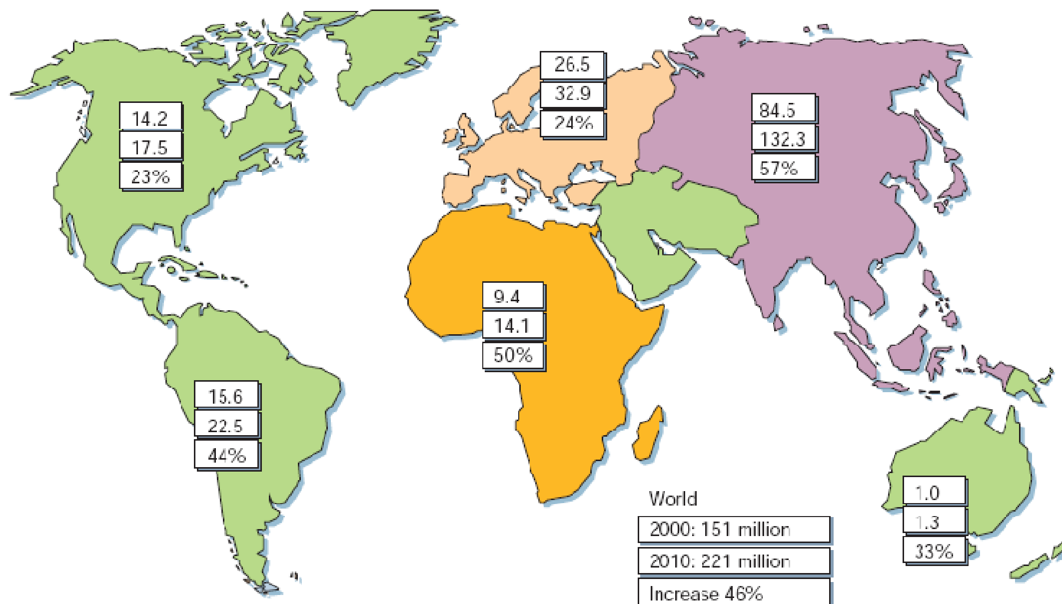

Figure 1. Nombre de personnes atteintes du diabète (en millions) en 2000 et 2010 (valeurs du haut et du milieu, respectivement) et le pourcentage d'augmentation (Zimmet et al, 2001).

1.2. Le diabète chez les populations autochtones

Chez les populations autochtones (Amérique, Océanie), le diabète prends des proportions encore plus importantes (Yu et al, 2007). Au Canada, on estime que 20% des autochtones sont touchés par le diabète, comparativement à 5% pour la population générale (Health Canada, 2002). Ce haut taux de diabète s'accompagne d'une prévalence élevée de surpoids et d'obésité qui touche deux fois plus les populations autochtones que la population générale (Garriguet, 2008). Ceci s'explique, premièrement, par une prédisposition génétique, l'environnement rude (style de vie nomade, hivers rigoureux, …) dans lequel ont évolué ces populations leur imposant de pouvoir stocker au maximum l'énergie à des fins de survie (Neel, 1999). Mais surtout, ce taux important d'obésité et de diabète serait du à un changement dans leur style de vie (Hegele, 2001). Ce sont des populations qui sont en effet passées d'un mode de vie nomade et traditionnel à une sédentarisation souvent forcée. Elles ont adopté les habitudes alimentaires, souvent riches en calories, de leurs voisins non autochtones. Les conséquences du diabète sont également aggravées par une faible acceptation des traitements et de la médecine moderne, principalement pour des raisons culturelles (Kuzmina et al, 2008).

1.3. Traitements actuels contre le diabète

Les différents traitements contre le diabète actuellement disponibles ont comme principal but la diminution de l'hyperglycémie. De nombreuses études montrent qu'en diminuant le taux de glucose sanguin, le risque de complications micro- et macrovasculaires diminue également (Diabetes Control and Complications Trial Research group, 1993; U.K Prospective Diabetes Study group, 1998). Il est possible de normaliser la glycémie d'un patient diabétique de plusieurs façons (Tableau 1).

Les agents sulfonylurés diminuent le taux de glucose sanguin en améliorant la sécrétion d'insuline par le pancréas (Panten et al, 1996). Ils agissent en se liant aux canaux potassiques ATP-dépendants de la membrane plasmique des cellules ß-pancréatiques. De ce fait, ils bloquent le flux de potassium à travers la membrane et entraînent une dépolarisation. Ceci entraîne l'ouverture des canaux calciques voltage-dépendants et l'entrée de calcium dans les cellules. L'accumulation de calcium intracellulaire favorise la fusion de granules d'insuline à la membrane plasmique et la sécrétion d'insuline par les cellules ß-pancréatiques.

Les médicaments de la classe des biguanides, dont la metformine est le plus connu, agissent en réduisant la gluconéogenèse hépatique et en augmentant la prise de glucose par les tissus périphériques (Giannarelli et al, 2003). Leur mécanisme d'action implique l'activation d'une enzyme intracellulaire de régulation de l'énergie, la protéine kinase activée par l'AMP (AMPK). Parmi les nombreuses fonctions attribuées à cette enzyme, il y a l'inhibition de l'expression de gènes hépatiques impliqués dans la gluconéogenèse (PEPCK, Glc-6-Pase) ainsi que le transfert de transporteurs de glucose (GLUT) du cytosole vers la membrane plasmique (tissus adipeux, musculaires,…) permettant l'entré de glucose dans la cellule (Leverve et al, 2003; Misra et al, 2007).

Les thiazolidinédiones (roziglitazone, pioglitazone) sont une classe de médicaments qui permettent d'améliorer la sensibilité des tissus périphériques à l'insuline chez une personne souffrant du diabète (Lehmann et al, 1995). Les thiazolidinédiones agissent principalement au niveau du tissu adipeux où ils se lient aux récepteurs nucléaires des acides gras, les PPARγ (*Peroxisome Proliferator-activated Receptor γ*). Une fois activés, ces récepteurs migrent vers l'ADN et augmentent la transcription de

certains gènes favorisant la différenciation des adipocytes, ce qui résulte en une redistribution des acides gras présents dans les muscles et le foie vers les adipocytes et une amélioration générale de la sensibilité à l'insuline (Hauner, 2002; Gelman et al, 2007).

Les inhibiteurs de DPP-4 (*Dipeptidyl Peptidase IV*) bloquent l'action de cette enzyme connue pour dégrader GLP-1 (*glucagon-like peptide 1*). GLP-1 est une incrétine, une hormone gastro-intestinale qui stimule la sécrétion d'insuline lorsque la glycémie est trop élevée. L'inhibition de DPP-4 entraîne donc l'augmentation de GLP-1 et la sécrétion d'insuline, tout comme le font les analogues ou mimétiques du GLP-1 tel l'exanatide (Richter et al, 2009).

Finalement, d'autres médicaments, tel l'acarbose, réduisent l'absorption intestinale de glucose (Hanefeld et al, 2008). L'acarbose se lie à l'enzyme α-glucosidase, une enzyme intestinale. En se liant à elle, l'acarbose diminue la capacité de celle-ci à digérer les glucides et par le fait même, moins de glucose est absorbé par le patient.

Ces traitements médicaux sont souvent accompagnés de moyens visant à diminuer le poids corporel (exercice, saine alimentation) souvent directement lié au développement du diabète de type II (Ross et al, 2000). Ces traitements sont, toutefois, souvent accompagnés d'effets secondaires indésirables. Les sulfonylurés ont une efficacité limitée et peuvent entraîner l'hypoglycémie (Tran et al, 2009). Les biguanides ont l'acidose métabolique comme effet secondaire potentiel (Luft et al, 1978). Réduire l'absorption intestinale de glucose peut entraîner des diarrhées chez le patient diabétique (Hillebrand et al, 1979). Il reste donc encore beaucoup à faire dans le développement de médicaments antidiabétiques avec effets secondaires minimes.

Tableau 1. Liste des principaux traitements antidiabétiques actuellement disponibles ainsi que le mode d'action de chacun.

Classe de médicaments antidiabétiques	**Mode d'action**
Sulfonylurés	Augmentation de la sécrétion d'insuline par le pancréas via le blocage des canaux potassiques ATP-dépendant
Biguanides	Diminution de la production hépatique de glucose et augmentation de la prise de glucose par les tissus périphériques (muscles, principalement)
Thiazolidinédiones	Augmentation de la sensibilité à l'insuline des tissus périphériques via l'activation des PPARγ
Inhibiteurs de DPP-4	Augmentation de la sécrétion d'insuline via l'augmentation de l'incrétine GLP-1
Acarbose	Réduction de l'absorption intestinale de glucose

1.4. Utilisation de la médecine traditionnelle dans le traitement du diabète

À travers le monde, beaucoup de populations diverses se fient encore à la médecine traditionnelle et ancestrale afin de traiter différentes maladies ou troubles de la santé. L'utilisation des plantes à des fins thérapeutiques date de la nuit des temps et les plantes sont et ont toujours été importantes dans le développement de nouveaux traitements ou molécules médicamenteuses (Farnsworth et al, 1985). À titre d'exemple, l'acide acétylsalicylique (le fameux médicament aspirine), un des médicaments les plus utilisé dans le monde pour ses propriétés analgésiques, antipyrétiques et anti-inflammatoires provient de l'acétylation de l'acide salicylique, acide ayant été isolé en premier dans l'écorce de saule (*salix* en latin) (Jeffreys, 2005). Autre exemple, le médicament anticancéreux Paclitaxel (Taxol) est un agent antimitotique provenant des feuilles du *Taxus brevifolia* (If de l'Ouest) et découvert dans les années 1960 (Wall et al, 1995). Il est aujourd'hui utilisé dans le traitement de différents cancers tels ceux de

l'ovaire, du poumon ou du sein. Dans le domaine du diabète, le médicament metformine, très largement utilisé, provient de l'espèce végétale *Galega officinalis* (Galéga officinale) (Cavaliere, 2007). Ces exemples montrent bien l'importance des végétaux comme pourvoyeurs de molécules à visées thérapeutiques.

Des plantes médicinales ayant des effets hypoglycémiants, et donc utiles dans le traitement du diabète, sont courantes dans la médecine traditionnelle de nombreuses populations à travers le monde (Haddad, 2006). Une meilleure compréhension de l'action de ces plantes peut aider à l'élaboration de remèdes antidiabétiques plus facilement acceptés par celles-ci. Cela peut également permettre l'élaboration de nouveaux traitements antidiabétiques possédant moins d'effets secondaires. Cependant, beaucoup reste à faire dans l'étude des effets et du mode d'action de ces diverses espèces végétales. L'équipe IRSC (Institut de Recherche en Santé du Canada) sur les médecines autochtones antidiabétiques, dont fait partie le laboratoire du Docteur Pierre Haddad au département de pharmacologie de l'Université de Montréal, s'intéresse à la médecine traditionnelle des Cris du nord du Québec. Le projet a pour but d'identifier des espèces végétales de leur pharmacopée traditionnelle ayant des propriétés antidiabétiques et ainsi aider ces populations dans leur combat quotidien contre le diabète, également devenu un problème de santé majeur chez elles. De plus, cette approche vise à contrer la déconnection culturelle des traitements de la médecine conventionnelle qui leur sont généralement offerts. Une étude ethnobotanique a permis l'identification de plusieurs espèces de plantes utilisées dans le traitement d'un quinzaine de symptômes reliés au diabète tels la polyurie, l'infection des plaies ou encore les douleurs aux extrémités (Leduc et al, 2006).

1.5. Plantes testées dans l'étude

Lors de cette première enquête ethnobotanique, les espèces végétales ont été identifiées à l'aide des ainés et guérisseurs de la communauté Cris de Mistissini. Pour cette étude, il s'agit d'*Abies balsamea* (L.) Mill. (sapin baumier), d'*Alnus incana* subsp. rugosa (Du Roi) R. T. Clausen (aulne rugueux), de *Larix laricina* K. Koch (mélèze laricin), de *Picea mariana* BSP (épinette noire), de *Pinus banksiana* Lamb. (pin gris), de *Rhododendron groenlandicum* (Oeder) Kron & Judd (thé du labrador), de *Sarracenia purpurea* L. (une plante carnivore) et de *Sorbus decora* C.K. Schneid. (sorbier décoratif)*. Ces plantes, ainsi que beaucoup d'autres utilisées dans les médecines traditionnelles des différents peuples autochtones de l'Amérique du Nord, ont déjà fait l'objet de plusieurs études (Clavelle, 1997; Moerman, 1998; Marles et al, 2000). Plusieurs plantes de la forêt boréale dont, *Abies balsamea*, *Picea Mariana* ou *Rhododendron groenlandicum* ont été étudiées pour leur potentiel antioxydant avec des résultats intéressants quant à leur capacité à neutraliser les radicaux libres de l'oxygène (McCune et al, 2002), un paramètre intéressant à considérer dans le traitement du diabète. Les Cris utilisent les différentes parties de chaque plante (feuilles, racines, écorces, résine, …) sous forme d'infusions, de pommades, de décoctions contre différents troubles de la santé allant du simple rhume aux brulures sérieuses et coupures profondes (Moerman, 1998). Les racines de plusieurs plantes sont utilisées comme nourriture et le bois d'une grande variété d'arbres sert à la confection de traineaux, canots ou tipis. Ces plantes ont donc une grande importance médicale et culturelle pour les Cris.

En laboratoire, pour chaque plante, les parties utilisées en médecine traditionnelle, qu'il s'agisse de l'écorce, des feuilles ou de la plante en totalité, ont été séparées du reste

*Dans cette nomenclature, le nom qui suit le nom latin de la plante et celui du botaniste qui en premier à décrit et classifié celle-ci.

de la plante, extraites à l'aide d'une solution de 80% d'éthanol aqueux et lyophilisées ensuite (Spoor et al, 2006). Les extraits poudreux ainsi obtenus sont solubilisés dans du *dimethyl sulfoxide* (DMSO) avant les traitements. Ces 8 extraits ont été étudiés, entre autres, pour leur capacité à améliorer la prise de glucose par des cellules musculaires ou à stimuler la sécrétion d'insuline par des cellules pancréatiques, des effets qui pourraient se traduire par une activité antidiabétique *in vivo* (Spoor et al, 2006). La concentration maximale non-toxique dans les cellules (musculaires et pancréatiques) a été déterminée pour chacun des extraits et les cellules ont été traitées pour 18 heures à cette concentration. Les effets des extraits ont été comparés à ceux du témoin véhicule (le DMSO) et à un témoin positif; dans le cas des essais sur la prise de glucose, à la metformine. Aucun des extraits n'a stimulé la sécrétion d'insuline par les cellules pancréatiques, suggérant que les extraits n'ont pas d'effets antidiabétiques au niveau des cellules β. Toutefois, parmi les 8 extraits, 6 ont montré une augmentation significative de la prise basale (en absence d'insuline) de glucose par les cellules musculaires, *Sarracenia purpurea* et *Sorbus decora* étant les extraits ayant eu les effets les plus importants. *Sarracenia purpurea* a même montré une augmentation encore plus importante que le médicament metformine. Ceci suggère un effet insulinomimétique des extraits, plutôt qu'un effet d'amélioration de la sensibilité à l'insuline, et que donc les extraits n'agissent pas via la voie de signalisation de l'insuline. En effet, aucun des extraits n'a montré une activation de la phospho-Akt (marqueur de la voie de signalisation du récepteur à l'insuline) lorsque mis en présence de cellules musculaires C2C12 (Martineau et al, 2009; voir annexe).

1.6. Mode d'action des extraits

Dans les essais de prise de glucose, la metformine a été utilisé comme contrôle positif. Tel que mentionné, la metformine est un médicament antidiabétique à prise orale de la famille des biguanides. C'est un médicament antidiabétique de premier choix dans le traitement du diabète de type II, surtout chez les personnes obèses (American Diabetes Association, 2009). Il possède très peu d'effets secondaires et permet en plus de diminuer le taux de cholestérol et de triglycérides circulants (Wulffele et al, 2004). La metformine permet surtout de réduire la production hépatique de glucose (Giannarelli et al, 2003), mais elle améliore également la sensibilité à l'insuline, la prise périphérique de glucose et l'oxydation des graisses (Lin et al, 2000; Bugianesi et al, 2005). Tel que mentionné plus haut, la metformine agit via l'activation intracellulaire de l'AMPK (Zhou et al, 2001). L'AMPK est une enzyme importante dans la régulation de la balance énergétique au niveau cellulaire, présente dans de nombreux tissus dont le foie, les muscles et le cerveau (Winder et al, 1999). Si la quantité d'ATP intracellulaire se trouve diminuée, ceci entraîne une augmentation du ratio AMP/ATP. C'est le signal qui permet l'activation de l'AMPK, qui aura pour but de rétablir la quantité d'ATP intracellulaire. Pour cela, l'AMPK va favoriser les fonctions permettant d'augmenter la production d'énergie par la cellule (Figure 2). Entre autre, l'AMPK va favoriser la translocation des transporteurs de glucose, les GLUT, du cytosol vers la membrane plasmique et permettre l'entrée de glucose dans la cellule (Ojuka et al, 2002).

Les GLUT sont une famille de protéines présente dans la majorité des tissus. Le glucose étant une molécule polaire (ne pouvant traverser la membrane lipidique cellulaire), les GLUT sont essentiels au transport du glucose à l'intérieur de la cellule. Il

existe plusieurs types de GLUT (numérotés de 1 à 12) divisés en plusieurs classes et ayant chacun une distribution particulière et un rôle spécifique dans le métabolisme glucidique (Thorens, 1996). Parmi les plus importants sont les GLUT4, présents dans le tissu adipeux, les muscles et le cœur. En condition physiologique normale, les GLUT4 sont séquestrés dans des vésicules à l'intérieur de la cellule. Lorsque l'AMPK est activé ou que l'insuline se lie à son récepteur au niveau de la membrane cellulaire, les vésicules migrent vers la membrane plasmique et fusionnent avec celle-ci (Lund et al, 1995; Watson et al, 2004). Les GLUT4 permettent alors la diffusion facilitée (ne nécessitant pas d'ATP) du glucose dans la cellule.

L'AMPK activé par la metformine favorisant la translocation des GLUT4 et l'entrée de glucose dans les cellules musculaires, ceci peut expliquer également les effets observés chez les cellules traitées avec les différents extraits. Les extraits ont en effet montré leur capacité à augmenter le contenu cellulaire en phospho-AMPK (AMPK activé) et ceci en corrélation directe avec l'augmentation de la prise de glucose (*Sarracenia purpurea* et *Sorbus decora* étant les plantes ayant le mieux activé l'AMPK) (Martineau et al, 2009).

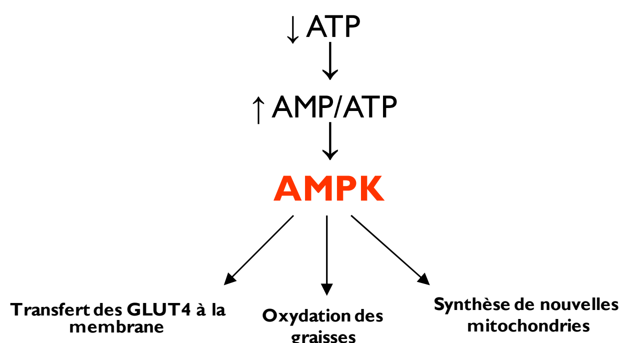

Figure 2. Schéma représentant le rôle de l'AMPK au niveau cellulaire. Une diminution de la quantité d'ATP intracellulaire fait augmenter le ratio AMP/ATP. C'est le signal d'activation pour l'AMPK qui va favoriser les réactions permettant de rétablir la balance énergétique de la cellule (transfert des GLUT4 à la membrane, oxydation des graisses, synthèse de nouvelles mitochondries, …)

1.7. Effets des extraits sur la respiration mitochondriale

Il est intéressant de constater que d'autres espèces de plantes (ou composés naturels provenant de ces plantes) que celles étudiées dans ce projet ont démontré la capacité d'activer l'AMPK. Elles agissent toutes sur la voie de production de l'énergie cellulaire, la chaîne respiratoire mitochondriale (Lee et al, 2006; Collins et al, 2007; Ahn et al, 2008). Ces espèces végétales utilisent ce procédé d'altération de la voie de synthèse de l'ATP comme un mécanisme de défense contre les attaques d'insectes ou autres microorganismes (Polya, 2003). Ceci suggère un mécanisme commun via lequel les plantes antidiabétiques agiraient. À titre d'exemple, la *Nigella sativa*, une plante provenant de régions et de latitudes très différentes que celles à l'étude ici, a également démontré un potentiel antidiabétique en agissant sur la chaîne respiratoire mitochondriale et en activant l'AMPK (Benhaddou-Andaloussi et al, 2010; voir annexe).

Les extraits, tout comme la metformine, agiraient donc au niveau de la membrane interne mitochondriale, perturbant la phosphorylation oxydative et diminuant, par le fait même, la production aérobique d'ATP. En condition aérobique, le pyruvate produit par la glycolyse (métabolisme anaérobique du glucose) est oxydé par le cycle de l'acide citrique, ce qui permet d'obtenir les coenzymes réduits NADH et QH_2 (Horton et al, 2002). La phosphorylation oxydative implique l'oxydation de ces deux coenzymes qui mène à la formation d'ATP. NADH et QH_2 sont oxydés (transfert de leurs électrons à l'oxygène) par la chaîne respiratoire de la mitochondrie au niveau de sa membrane interne. Cette chaîne est composée de cinq complexes enzymatiques (I à IV et ATP synthétase). En traversent les complexes I à IV, les électrons favorisent le transfert de protons (H^+) de la matrice vers l'espace intermembranaire en passant via les complexes.

Il se produit ainsi un gradient de protons, avec la matrice plus alcaline et négativement chargée que l'espace intermembranaire. Les protons reviennent donc dans la matrice en passant par l'ATP synthase, une réaction couplée à la formation d'ATP à partir de l'ADP.

La phosphorylation oxydative peut être perturbée soit en découplant soit en inhibant la chaîne respiratoire. Les composés synthétiques appelés découpleurs (tel le 2,4-Dinitrophenol) stimulent l'oxydation des substrats en absence d'ADP, découplant ainsi les réactions d'oxydation de la production d'ATP (Heytler, 1979). Les découpleurs agiraient en transportant les protons de l'espace intermembranaire vers la matrice, empêchant le gradient de protons de repasser via l'ATP synthase (Figure 3). Les inhibiteurs (tel l'oligomycine), quant à eux, altèrent la phosphorylation oxydative en inhibant les complexes enzymatiques (Joshi et al, 1991). La metformine, par exemple, agit en inhibant le complexe I de la chaîne respiratoire (El-Mir et al, 2000; Owen et al, 2000). Quant aux extraits, certains ont montré simplement un effet inhibiteur, d'autres une combinaison d'effets inhibiteurs et découpleurs lorsque testés sur des mitochondries isolées de foie (Martineau et al, 2009). Ces effets sur la chaîne respiratoire ont entrainé une diminution de la production d'ATP et une activation de l'AMPK par les extraits. Toutefois, *Sarracenia purpurea* et *Sorbus decora*, qui ont démontré la meilleure activation d'AMPK et la meilleure prise basale de glucose par les cellules musculaires, n'ont pas été les extraits ayant le plus affecté la synthèse d'ATP, suggérant qu'une altération importante de la synthèse d'ATP n'est pas nécessaire à l'activation de l'AMPK.

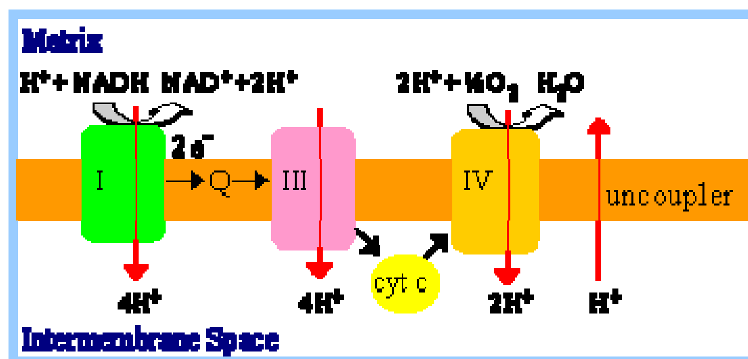

Figure 3. Les découpleurs bloquent la phosphorylation oxydative en transportant les protons contre le gradient (Diwan, 2007). En traversent les complexes I à IV, les électrons favorisent le transfert de protons (H^+) de la matrice vers l'espace intermembranaire en passant via les complexes. Les découpleurs transportent les protons de l'espace intermembranaire vers la matrice, empêchant le gradient de protons de repasser via l'ATP synthase, découplant ainsi les réactions d'oxydation de la production d'ATP.

1.8. Liens entre obésité et diabète

En plus de permettre la translocation des GLUT à la membrane, l'AMPK va favoriser de nombreuses autres fonctions cellulaires ayant pour but une augmentation de la production d'ATP. Parmi ces fonctions, il y a l'oxydation des graisses (Woo Je Lee et al, 2005). Tel que mentionné plus haut, les populations autochtones du Canada sont environ quatre fois plus touchés par le diabète que la population générale et leur taux d'obésité est le double de celui des non-autochtones (Health Canada, 2002; Garriguet, 2008). Il existe un lien étroit entre l'obésité et le diabète. L'obésité est considérée comme la principale cause du développement de l'insulino-résistance (Muoio et al, 2008). Un des facteurs les plus importants semble être la distribution de la graisse corporelle, une personne ayant beaucoup de graisse au niveau de l'abdomen étant plus susceptible de développer une résistance à l'insuline (Bokyo et al, 2000). Les adipocytes (cellules graisseuses), en nombre important chez les personnes obèses, produisent et sécrètent des adipokines, un groupe d'hormones et de cytokines qui régulent la production et

l'utilisation d'énergie par les tissus périphériques (Muoio et al, 2008). Parmi les adipokines les plus importantes il y a les hormones « anti-diabétogéniques » leptine et adiponectine et les médiateurs pro-inflammatoires interleukine 6 (IL-6) et TNFα (*tumor necrosis factor* α). La leptine agit essentiellement sur l'hypothalamus et diminue ainsi l'appétit permettant de réguler les réserves de graisse dans l'organisme. Les adiponectines jouent un rôle important dans la régulation du métabolisme des lipides et du glucose. La leptine et l'adiponectine ont également des fonctions de diminution de la synthèse des triglycérides, de stimulation de la β-oxydation des acides gras et d'augmentation de la sensibilité à l'insuline dans les muscles squelettiques et le foie. Chez les personnes obèses et résistantes à l'insuline, il a été noté que les taux de leptine sont augmentés et les taux d'adiponectine sont diminués suggérant que l'obésité mène à un état de résistance à la leptine et de déficience en adiponectine (Wannamethee et al, 2007; Li et al, 2010). Une diète riche en gras est également responsable de l'activation de la kinase IκB-β (IKKβ), une kinase qui active le facteur de transcription NF-κB (*nuclear factor kappa-light-chain-enhancer of activated B cells*) et augmente la production d'IL-6 et de TNFα. L'augmentation de ces médiateurs de l'inflammation chez les personnes obèses a également été associée au développement de l'insulino-résistance (Cai et al, 2005; Tilg et al, 2008).

Finalement, chez une personne en surpoids ou obèse, il existe des taux importants d'acides gras circulants (Patersen et al, 2006; Timmers et al, 2008). Ces acides gras finissent par s'accumuler dans des tissus périphériques, tels les tissus musculaires ou hépatiques (Figure 4). Là, ils sont métabolisés, via l'*Acyl-CoA synthetase*, en *acyl-CoAs à longue chaîne* (LCACoAs). Ces LCACoAs peuvent soit être transportés dans les

mitochondries par la *Carnitine acyltransferase* pour y être oxydés (β-oxydation des acides gras), soit utilisés comme intermédiaires à la production de composés lipidiques tels que les diacylglycérols (DAG) ou encore les céramides.

L'insuline est une hormone centrale dans la régulation du métabolisme glucidique dans l'organisme. Elle est produite par le pancréas, une glande mixte (activités endocrine et exocrine) abdominale et reliée au tube digestif, plus spécialement par les cellules β des îlots de Langerhans. L'insuline a comme principale fonction la diminution de la glycémie via la prise de glucose par les tissus périphériques (muscles, tissus adipeux, cerveau, …) et l'inhibition de la glycogénolyse et de la gluconéogenèse hépatique (Chang et al, 2004). Par son action, elle s'oppose au glucagon, autre hormone produite et sécrétée par le pancréas (cellules α), qui lui agit en augmentant le taux de glucose sanguin lorsque nécessaire (White, 1999). La balance entre l'activité de ces deux hormones est donc nécessaire au bon fonctionnement de l'organisme. Le signal responsable de la sécrétion d'insuline par le pancréas est l'entrée de glucose dans les cellules β via les transporteurs GLUT2. Une fois dans la cellule, le glucose est métabolisé (glycolyse, chaîne respiratoire) ce qui entraîne une augmentation de la production d'ATP. Cette augmentation du taux d'ATP entraîne la fermeture des canaux potassiques ATP-dépendants, une dépolarisation, l'entrée de calcium dans le cytosol et la relâche d'insuline dans le sang (mécanisme similaire à celui des sulfonylurés) (Rasmussen et al, 1990). Lorsque l'insuline sécrétée par le pancréas se lie à son récepteur au niveau cellulaire, ceci entraîne l'autophosphorylation en tyrosine du récepteur (Timmers et al, 2008). Le récepteur ainsi activé recrute différentes molécules, dont les protéines IRS (*Insulin Receptor Substrat*) qu'il phosphoryle également en tyrosine. Il s'ensuit une

cascade de signalisation avec la phosphorylation et l'activation de PI3K (*Phosphatidylinositol 3-kinases*) et de PKB/Akt (Protéine Kinase B), ce qui permet, dans les cellules musculaires, le transfert des GLUT (principalement les GLUT 4) du cytosol vers la membrane plasmique et l'entrée du glucose dans la cellule. Dans les cellules hépatiques, l'activation de PI3K et d'Akt entraîne une diminution de la gluconéogenèse et l'activation de la *glycogène synthétase* menant à la synthèse de glycogène (forme de stockage de glucose), tout ceci dans le but de diminuer le taux de glucose sanguin.

Les DAG qui s'accumulent dans la cellule suite à l'entrée massive des acides gras sont soit métabolisés en triglycérides (TG), des composés inertes comparables au glycogène (forme de stockage des acides gras cette fois-ci), soit ils activent les protéines kinase C (PKC). Les PKC favorisent la phosphorylation en sérine/thréonine, plutôt qu'en tyrosine, du récepteur à l'insuline et des protéines IRS (Timmers et al, 2008). Le récepteur n'est ainsi plus capable de répondre à la liaison de l'insuline et à activer la cascade de signalisation. Les céramides affectent également la voie de signalisation de l'insuline, plus en aval, en inhibant la phosphorylation de l'Akt. Ceci entraîne donc une résistance à l'insuline qui, si elle devient chronique, mènera éventuellement au développement du diabète de type II chez les personnes en surpoids.

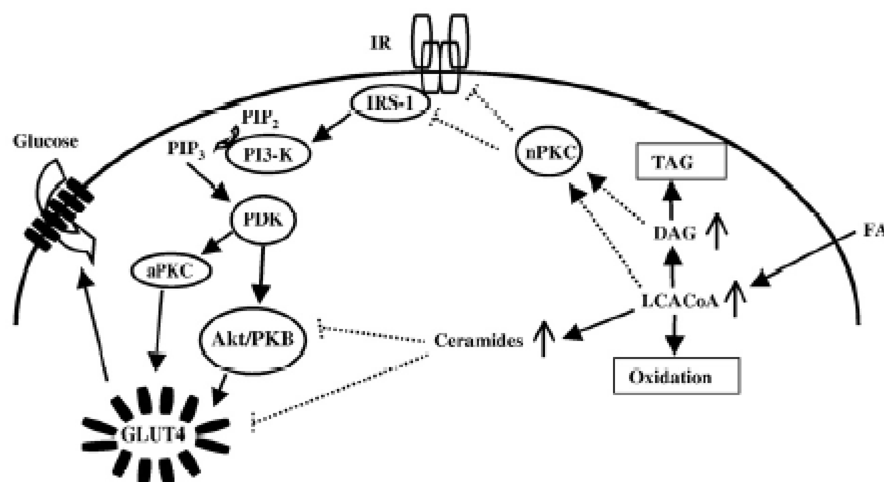

Figure 4. Relation entre l'accumulation d'acides gras libres et le développement de l'insulino-résistance dans le tissu musculaire (Timmers et al, 2008). L'accumulation d'acides gras (FA) dans la cellule musculaire entraîne l'augmentation de composés tels les nPKC ou les céramides qui inhibent la voie de signalisation de l'insuline (résistance à l'insuline) entraînant une diminution du transfert des GLUT4 à la membrane et le développement de l'hyperglycémie.

1.9. Buts du Projet

Tel que déjà mentionné, les extraits provenant de plantes médicinales utilisées dans la pharmacopée Cri ont montré une amélioration de la prise basale de glucose par les cellules musculaires, ceci en lien avec une activation de l'enzyme AMPK (Spoor et al, 2006; Martineau et al, 2009). Également, les extraits ont majoritairement démontré des effets (découpleurs ou inhibiteurs) sur la chaîne respiratoire de mitochondries isolées. Notre premier objectif était donc de mesurer l'intensité du stress métabolique que ces mêmes extraits peuvent induire au niveau de cellules entières en culture, qu'elles soient de type musculaire ou hépatique, stress qui serait responsable de l'activation de l'AMPK. En effet, si les extraits interfèrent avec la voie normale de production d'énergie, ceci entraîne un stress métabolique cellulaire avec une diminution du taux d'ATP intracellulaire et une augmentation de l'utilisation de la voie anaérobique de production

d'énergie (la glycolyse). L'utilisation accrue par la cellule de la glycolyse entraîne la production d'acide lactique (Horton et al, 2002). Comme il est dangereux pour celle-ci d'accumuler de l'acide lactique, elle s'en débarrasse dans le milieu extracellulaire, acidifiant ce même milieu par l'accumulation de protons. En mesurant le taux d'acidification du milieu extracellulaire (changement de pH) suite aux traitements avec les extraits, nous tentons de vérifier indirectement l'effet des extraits sur la respiration mitochondriale. Également, en mesurant de façon plus directe le taux d'ATP intracellulaire suite aux traitements, nous avons une autre idée du stress métabolique subit par les cellules.

Deuxièmement, comme l'AMPK permet l'oxydation des graisses et que la présence de ces graisses au niveau musculaire et hépatique est un facteur important de développement de résistance à l'insuline, nous avons voulu étudier l'effet des extraits sur un modèle hépatique de résistance à l'insuline induite par les graisses. Pour cela, nous avons traité des cellules hépatiques H4IIE avec un acide gras saturé, le palmitate, en espérant entraîner une accumulation de lipides dans les cellules et que cette accumulation perturbe la voie de l'insuline. Dans cette étude, nous voulions voir si les extraits ont la capacité de réduire le contenu en lipides des cellules (mesure du contenu en triglycérides) et si cette réduction se traduit par une normalisation de la voie de signalisation de l'insuline (mesure de la phosphorylation d'Akt après traitement à l'insuline).

2. Matériel et méthodes

2.1. Culture cellulaire

Les différentes expérimentations ont lieu sur des cellules en culture. Il s'agit de cellules musculaires (myoblastes) squelettiques C2C12 provenant de souris et de cellules hépatiques H4IIE provenant de rats. Les cellules sont obtenues de la compagnie ATCC (*American Type Culture Collection*; Manassas, VA). Les cellules sont incubées à 37° C en présence de 5% CO_2 (incubateur stérilisé). Les myoblastes sont proliférés jusqu'à confluence dans du milieu DMEM (*Dulbecco's modified eagle medium*) enrichi de glucose et supplémenté avec 10% FBS (*fetal bovine serum*), 10% HS (*horse serum*) de même que des antibiotiques. Puis, les myoblastes à confluence sont différenciés en myotubes plurinucléaires pour une période de 7 jours avant le début des essais dans du milieu DMEM enrichi de glucose et supplémenté de 2% HS et des antibiotiques. Les hépatocytes sont proliférés jusqu'à confluence dans du milieu DMEM enrichi de glucose et supplémenté avec 10% FBS et des antibiotiques. Les expérimentations ont lieu le jour où les cellules atteignent 100% confluence ou au plus 1 jour après confluence totale. Le milieu de culture DMEM enrichi de glucose provient de la compagnie *Wisent* (St-Bruno,QC). Le FBS, le HS et les antibiotiques sont fournis par la compagnie *Sigma-Aldrich* (Oakville, ON).

2.2. Test de détection du changement de pH

Un test spectrophotométrique de mesure du changement de pH du milieu de culture cellulaire en fonction du temps, quantitatif entre pH 7,2 et 6,4, a été mis au point en se basant sur des expérimentations similaires (Schornack et al, 2003; Yang et al, 2003). Cet essai permet d'évaluer l'acidification du milieu extracellulaire, synonyme

d'augmentation de la glycolyse et du stress métabolique, induit par les différents extraits. Un tampon à base de phosphate, le D-PBS (*Dulbecco's phosphate buffered saline*), a été préparé et du rouge phénol, qui sert d'indicateur de pH, y a été rajouté. La recette originale du D-PBS a été modifié pour ne contenir que 2mM de phosphate, 5 fois moins que dans la recette originale et ceci afin d'avoir la possibilité de mesurer de faibles changements de pH (diminution de la capacité de tamponnage du D-PBS). Les sels composant ce tampon D-PBS modifié (mD-PBS) ont été balancés pour compenser la réduction du sodium et du potassium associés au phosphate et ainsi maintenir l'osmolarité physiologique normal du milieu dans lequel baignent les cellules (recette du mD-PBS donnée plus bas). Schornack et Gillies ont découvert qu'à pH très acide (pH=3), le rouge phénol a un maximum d'absorbance à 435 nm et à pH très basique (pH=11) un maximum d'absorbance à 550 nm. Ils ont ainsi déterminé que le pH était fonction du ratio Abs550/Abs435. Dans le cadre de ce protocole, la relation entre l'Abs530/Abs450 et le pH pour le tampon mD-PBS a été calculée et ceci a donné une relation linéaire entre pH 6,4 et pH 7,4 pouvant être déterminée avec la formule : pH = 0,765 x ln (abs 530/abs 450) + 7,61 (R^2 = 0,99). Puis, la capacité de tamponnage du mD-PBS a été déterminée en établissant une relation entre la quantité d'OH^- rajouté (mM) et le pH. Cette relation s'est avéré être linéaire entre pH 6,2 et pH 7,2 (1,075 mM OH^-/unité pH) et il a donc été décidé que le pH de départ pour les expérimentations serait 7,2 (pH pas très loin de 7,4 et donc peu nuisible pour les cellules). Le pH de ce tampon étant de 7,1 suite à sa préparation, il est ajusté à 7,2 à température ambiante avec du NaOH peu avant le début de chaque test.

Les expériences sont réalisées sur des cellules en culture dans des plaques de 12 puits (*Sarstedt Inc.*, Montréal, QC). Les cellules musculaires C2C12 sont traitées 7 jours

après différenciation, tandis que les hépatocytes H4IIE sont traités 1 jour après confluence totale (100%). Le jour de l'expérimentation, les cellules sont rincées deux fois avec le tampon, puis 1,0 ml de tampon est ajouté à chaque puits et les cellules sont maintenues 30 minutes à 37ºC dans un incubateur sans CO_2 (permet aux cellules de s'équilibrer au tampon). Un incubateur sans CO_2 est nécessaire car, le tampon s'est avéré sensible au CO_2 (acidification du tampon) ce qui influe sur les résultats. Le test débute en mixant doucement les traitements préparés à 3 fois la concentration désirée dans un volume de 500 µl de mD-PBS et préchauffés à 37ºC aux 1,0 ml de mD-PBS déjà présents dans les puits pour un volume final de 1,5 ml et les traitements aux concentrations voulues. Les extraits sont testés à leur concentration maximale non-toxique telle que déterminée dans les cellules C2C12. Puis, 100 µl de milieu, correspondant au temps 0, sont prélevés et transféré à une plaque de 96 puits (*Sarstedt Inc.*, Montreal, QC) pour l'analyse spectrophotométrique (absorbance à 530 nm suivi de l'absorbance à 450 nm). Les cellules sont ensuite incubées à 37ºC dans un incubateur sans CO_2 pour la durée de l'expérience. Aux temps 20, 40, 60, 120, 180 et 240 minutes, les plaques sont retirées de l'incubateur et 100 µl de milieu sont prélevés pour analyse spectrophotométrique. Le calcul du taux d'acidification et du taux cumulatif de sécrétion d'équivalents acides en fonction du temps tien compte de la diminution de volume du milieu suite à chaque prélèvement. Puisque le DMSO, habituellement utilisé comme témoin véhicule, a démontré la capacité à stimuler l'acidification, comme d'autres l'ont noté (Gerber et al, 1996), les extraits sont initialement solubilisés dans 80% éthanol (le même solvant utilisé pour l'extraction des espèces). Au moment des essais, la concentration finale d'éthanol, nouveau témoin véhicule, est de 0,08%. L'addition des extraits ou contrôles affectant le

pH du tampon, tous les traitements sont ajustés séparément à pH 7,2 peu avant le début de l'expérience. Le découpleur FCCP (*Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone*; *Sigma-Aldrich*, Oakville, ON) solubilisé dans de l'éthanol est utilisé comme contrôle positif à une concentration de 5 µM.

Recette pour 1 litre de tampon mD-PBS 1X

$CaCl_2$	2 mM	(PM=147,02)	294 mg
KCl	4 mM	(PM=74,56)	298 mg
KH_2PO_4	0,5 mM	(PM=136,09)	68 mg
$MgCl_2$	2 mM	(PM=203,3)	407 mg
NaCl	137 mM	(PM=58,44)	8006 mg
Na_2HPO_4	1,5 mM	(PM=268,07)	402 mg
Glucose	25 mM	(PM=180,2)	4505 mg
Rouge Phénol*	0,1 mM	(solution 0,5%)	7,1 ml

* *Sigma-Aldrich*, Oakville, ON

2.3. Mesure de l'ATP cellulaire

Si le mode d'action des extraits consiste à affecter la chaine respiratoire mitochondriale, ceci pourrait se traduire par une diminution de l'ATP intracellulaire. Afin de vérifier cette diminution, un kit de détection de l'ATP luminescent (ATPLite) de la compagnie *Perkin Elmer* (Waltham, MA) a été utilisé. Le principe de ce kit consiste en une réaction entre l'ATP, la D-luciférine et la luciférase. La luciférase catalyse la réaction entre l'ATP et la D-luciférine, ce qui se résulte en une production de lumière. La lumière émise est quant à elle proportionnelle à la concentration d'ATP. Le kit d'ATPLite est composé d'une solution de lyse des cellules de mammifère, d'une solution tampon du

substrat, du substrat (luciférase/luciférine) et d'ATP pour l'étalonnage. Dans le cas de l'ATPLite, les hépatocytes sont directement ensemencés dans des plaques de 96 puits (100 µl de suspension cellulaire par puits), tandis que les cellules C2C12 sont ensemencées dans des plaques de 24 puits (500 µl de suspension cellulaire par puits) car, elles se différencient difficilement dans des plaques de 96 puits. Puis, elles sont traitées avec les différentes conditions désirées pour 1, 3 et 6 heures. DMSO 0,1% sert de témoin véhicule tandis que FCCP 5 µM sert de contrôle positif. Suite aux traitements, 50 µl de solution de lyse sont ajoutés aux 100 µl de suspension cellulaire (pour les C2C12, les volumes sont augmentés proportionnellement : 250 µl de solution de lyse sont ajoutés aux 500 µl de suspension cellulaire) et la plaque est agitée 5 minutes à 700 rotations par minute (rpm). La solution de lyse permet la lyse des cellules en plus d'une augmentation du pH qui permet l'inactivation d'enzymes endogènes de dégradation de l'ATP, les ATPases. Pendant les 5 minutes de rotation de la plaque, le substrat est mis en solution par l'ajout de 5 ml de solution tampon. Puis 50 µl de solution reconstituée sont rajoutés aux puits et la plaque est agitée 5 minutes à 700 rpm. Ceci ramène le pH au niveau physiologique et permet à la réaction d'avoir lieu. La plaque est finalement privée de lumière pour 10 minutes et la luminescence est mesurée à l'aide du spectrophotomètre. L'ATP fourni dans le kit permet d'obtenir une courbe standard afin de quantifier l'ATP à partir des valeurs de luminescence. La courbe d'ATP standard est préparée en parallèle à l'expérimentation principale.

2.4. Détermination de la concentration maximale non-toxique des extraits dans les hépatocytes H4IIE

Pour les essais de changement de pH et de mesure de la quantité d'ATP décrits plus haut, les extraits ont été testés à leurs concentrations maximales non-toxiques telles

que déterminées dans les cellules musculaires C2C12. Toutefois, comme les essais sur les effets des extraits sur des cellules devenues résistantes à l'insuline ont lieu sur des hépatocytes H4IIE, il était nécessaire de déterminer la concentration maximale non-toxique de chaque extrait pour ce type cellulaire qui peut être différente de celle mesurée dans les cellules C2C12. Pour cela, chaque extrait a été testé à une concentration quatre fois supérieure à celle obtenue dans les cellules C2C12, sur une période de 24 heures, puis les concentrations ont été progressivement diminuées selon qu'elles montraient ou non de la toxicité. Dès qu'une concentration ne montrait pas de toxicité, elle était répétée deux autres fois (n=3) afin d'être confirmée. La toxicité a été déterminée selon le ratio de lactate déshydrogénase (LDH) relâchée par les cellules dans le milieu extracellulaire sur la quantité totale de LDH. Ce ratio a été comparé avec celui des cellules traitées simplement avec le témoin véhicule (DMSO) pour chaque extrait. La LDH est une enzyme qui est libérée du cytosol lorsqu'il y a un dommage à la membrane cytoplasmique. En la quantifiant dans le milieu extracellulaire, il est possible de déterminer s'il y a eu ou non de la toxicité cellulaire suite au traitement. Afin de pouvoir quantifier la LDH, un « kit » de détection de LDH provenant de la compagnie *Roche* (Mannheim, Allemagne) a été utilisé. Le principe de l'essai est que la LDH relâchée dans le milieu extracellulaire, en catalysant l'oxydation du lactate en pyruvate, réduit le NAD^+ en $NADH+H^+$. À l'étape suivante, le $NADH+H^+$ réduit (transfert de $2H^+$) le sel tetrazolium (de couleur jaune) en formazan (de couleur rouge). Donc, plus la solution est rouge, plus il y a eu de relâchement de LDH par les cellules (lien direct).

2.5. Préparation du palmitate

Le palmitate est un acide gras saturé souvent utilisé en laboratoire pour mimer les effets d'une diète grasse au niveau cellulaire (Mordier et al, 2007; Nakamura et al, 2009). L'accumulation de palmitate dans les cellules hépatiques peut entraîner de la stéatose, de l'inflammation et même altérer la voie de signalisation de l'insuline. Afin que le palmitate puisse s'accumuler dans les hépatocytes, il faut le coupler à une protéine de transport, comme l'albumine de sérum bovin (BSA). Une concentration stock de 4mM palmitate/5% BSA est préparée. Tout d'abord, des solutions de Krebs-Ringer Buffer (KRB) 5X (recette donnée plus bas), de $NaHCO_3$ 100 mM et de Hepes 1 M à pH 7,4 sont préparées. À partir de ces solutions est préparé du Krebs-Ringer Buffer + Hepes (KRBH) 1X (KRB 1X, $NaHCO_3$ 2 mM et Hepes 10 mM) auquel est rajouté 5% de BSA. Le pH de cette solution est ajusté à 7,4 (avec NaOH ou HCl) après 1 heure d'incubation à 37° C en présence de 5% CO_2. À 30 ml de cette solution sont rajoutés 200 mg de sel de sodium palmitate. Le palmitate est laissé à dissoudre dans la solution de KRBH-5% BSA pour la nuit à 37° C avec agitation lente. Le lendemain, cette solution est filtrée (il est impossible de pouvoir dissoudre la totalité du palmitate) et la concentration de palmitate est mesurée grâce à un « kit » de mesure des acides gras provenant de la compagnie *WAKO* (Richmond, VA). La concentration obtenue se situe généralement entre 4,5 et 6,5 mM et la solution est diluée jusqu'à 4 mM avec le restant de KRBH-5% BSA. Le stock de palmitate 4 mM/5% BSA est dilué jusqu'à 150 µM lors des traitements sur les cellules.

Recette pour 1 litre de KRB 5X

Produit	P.M. (g/mol)	Molarité (mM)	Quantité à peser pour 1 litre (g)
NaCl	58,44	675	39,44
KCl	74,56	18	1,34
NaH_2PO_4	137,99	2,5	0,34
$MgCl_2$	203,3	2,5	0,5
$CaCl_2$	147,02	7,5	1,1

2.6. Dosage des triglycérides intracellulaires par AdipoRed

La prise de palmitate par les hépatocytes entraîne une accumulation de triglycérides. Le réactif lipophile AdipoRed, une solution de rouge de Nile dans du DMSO, entre spécifiquement dans les gouttelettes lipidiques et devient fluorescent à une longueur d'onde de 572 nm suite à une excitation à une longueur d'onde de 485 nm. Il est ainsi possible de quantifier la quantité de triglycérides accumulée dans les cellules suite à un traitement au palmitate. Suite à un traitement de 18 heures des cellules avec 150 μM palmitate (+ ou – les extraits à leur concentration maximale non-toxique dans les hépatocytes ou le contrôle positif) ou KRBH-5% BSA (témoin véhicule), les cellules sont rincées avec du tampon PBS. Les cellules sont mises en culture dans des plaques de 24 puits (*Sarstedt Inc.*, Montréal, QC) et traitées le jour où elles atteignent 100% de confluence. Le médicament antidiabétique metformine à une concentration de 1 mM sert de contrôle positif. Suite au rinçage, le réactif Adipored (*Lonza*, Walkersville, MD) est

ajouté aux cellules qui sont incubées 10-15 minutes à température pièce. La mesure de la fluorescence au spectrophotomètre est proportionnelle à l'accumulation de triglycérides dans les cellules.

2.7. Mesure de la fluorescence de phospho-Akt (ELISA)

La mesure de l'activité de l'Akt, enzyme clé de la voie de signalisation de l'insuline, est obtenue grâce à un test ELISA dont le protocole est fourni par la compagnie *R&D Systems* (Minneapolis, MN). Les cellules H4IIE sont mises en culture dans des plaques de 96 puits noires à fond clair. Le jour où elles atteignent 100% de confluence, les cellules sont traitées selon les différentes conditions en parallèle avec celles traitées pour le test de l'AdipoRed. Après 18 heures de traitement, les cellules sont rincées puis mises en présence de 10 nM d'insuline. Après une incubation de 15 minutes à 37° C, les cellules sont fixées avec du formaldéhyde 4% pour 20 minutes. Puis, les cellules sont perméabilisées avec du peroxyde d'hydrogène (H_2O_2) 0,6% à nouveau pour 20 minutes. Les cellules sont ensuite bloquées 1 heure avec 10% FBS (*fetal bovine serum*). Entre chaque étape, les cellules sont rincées plusieurs fois avec un tampon de lavage. Suite au blocage des cellules, celles-ci sont rincées plusieurs fois, puis mises en présence d'anticorps primaires anti-phospho-Akt (provenant de lapin) et anti-Akt total (provenant de souris) pour la nuit à 4° C. Le lendemain matin, les cellules sont rincées, puis les anticorps secondaires anti-lapin et anti-souris sont ajoutés. Les cellules sont incubées 2 heures à température pièce. Les anticorps secondaires sont conjugués à la *horseradish-peroxidase* (HRP) dans le cas de l'anticorps anti-lapin et à la phosphatase alcaline (AP) dans le cas de l'anticorps anti-souris. Après les 2 heures d'incubation en présence des anticorps secondaires, les cellules sont rincées plusieurs fois, puis le substrat

fluorescent pour HRP est ajouté. Les cellules sont incubées 1 heure à température pièce en étant protégées de la lumière. Ce substrat (F1) est excité à une longueur d'onde de 540 nm et émet de la fluorescence à une longueur d'onde de 600 nm. Une heure après l'ajout du premier substrat, le substrat fluorescent pour l'AP est ajouté et les cellules sont incubées 20 minutes à température pièce en étant à nouveau protégées de la lumière. Le deuxième substrat (F2) est excité à une longueur d'onde de 360 nm et émet de la fluorescence à une longueur d'onde de 450 nm. Les substrats F1 et F2 sont ajoutés séparément car leur ajout simultané nuirait au développement de la fluorescence. Les fluorescences émises sont mesurées au spectrophotomètre.

2.8. Analyse statistique

Les résultats sont exprimés en moyenne ± l'erreur type avec le nombre d'expériences indépendantes et le nombre de réplicas indiqués. Les données sont analysées par ANOVA (analyse de la variance) à un facteur à l'aide du logiciel *StatView* 5.0 (Institut SAS, Cary, NC). La différence significative a été établie à $p \leq 0,05$.

3. Résultats

3.1. Stimulation du taux d'acidification du milieu extracellulaire par les extraits

Le test de détection d'un changement de pH du milieu extracellulaire indique une production d'acide lactique par la cellule ayant augmenté son recours à la glycolyse anaérobique suite à la diminution de sa capacité de synthèse mitochondriale de l'ATP. Ce test a été développé au laboratoire du Docteur Haddad comme un moyen de vérifier de façon indirecte les effets des différents extraits sur la chaîne respiratoire de la mitochondrie. Cet essai a été utile en laboratoire non seulement pour mesurer les effets des extraits de plantes Cris, mais également de ceux d'autres plantes ou composés au potentiel antidiabétique telles la *Nigella sativa* et la quercétine (voir les articles traitant de ces sujets en annexe).

En ce qui concerne les effets des extraits de plantes Cris, cet essai, en mesurant le flux de protons, donne de l'information sur le degré de stress métabolique induit par les extraits ainsi que la rapidité et la durée de cette action. Il permet également de comparer les effets des extraits sur deux types cellulaires différents, les expérimentations ayant lieu sur des cellules musculaires C2C12 ainsi que sur des hépatocytes H4IIE. Les cellules sont mises en présence des extraits pour une période de 4 heures avec prélèvement du milieu extracellulaire à différents temps dans le but de mesurer le pH par spectrophotométrie (le phénol rouge sert d'indicateur). Les résultats sont exprimés sous la forme d'une sécrétion cumulative de H^+ en fonction du temps (figure 5) et représentent la moyenne de 4 à 5 réplicas provenant de deux expériences indépendantes (n=2). Vu le faible nombre d'expériences indépendantes, il n'a été possible de faire une analyse statistique des

résultats obtenus et il n'est donc pas possible de discuter de leur différence significative. Toutefois, il est possible de noter certaines tendances et de faire certaines interprétations (Martineau et al, 2009; voir annexe).

Ces résultats semblent montrer que dans les cellules C2C12, la majorité des extraits augmentent le taux d'acidification de 2 à 3 fois dans les 20 premières minutes d'incubation. Toutefois, les effets des différents extraits varient grandement après ce temps. *Abies balsamea*, *Larix laricina*, *Picea mariana* et *Pinus banksiana* sont les quatre extraits à montrer une augmentation continue du taux d'acidification sur une période de 4 heures. Au contraire, les effets d'*Alnus incana* ne durent pas plus de 20 minutes. Les autres extraits ne montrent que peu d'effets sur le taux d'acidification du milieu extracellulaire.

Dans les hépatocytes H4IIE, de légères différences avec les cellules musculaires C2C12 ont été notées. Notamment, *Picea mariana* n'a aucun effet sur le taux d'acidification dans les cellules H4IIE, contrairement à ce qui est observé dans les cellules C2C12. D'un autre côté, l'effet d'*Abies balsamea* semble beaucoup plus marqué dans les cellules H4IIE que dans les cellules musculaires C2C12, dépassant même l'effet du contrôle FCCP.

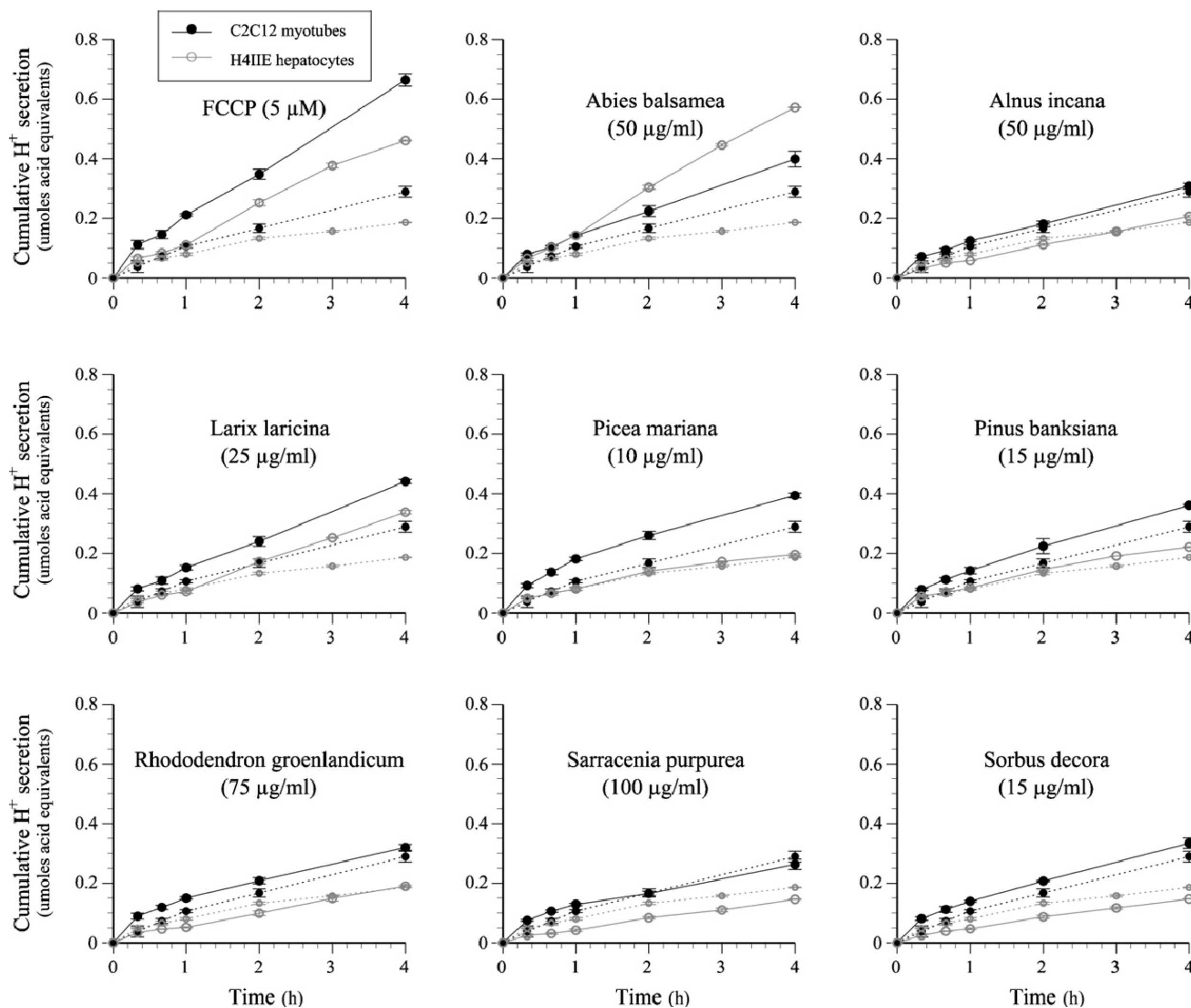

Figure 5. Sécrétion d'équivalents acides dans le milieu de culture, indice du taux de glycolyse anaérobie, selon chaque traitement avec les différents extraits dans les cellules C2C12 (cercles pleins) et les cellules H4IIE (cercles ouverts). Le découpleur FCCP est utilisé comme contrôle positif. Le témoin véhicule est représenté en lignes pointillées dans chaque graphique. Les valeurs exprimées sont des moyennes (4 ou 5 réplicas par condition et par temps) de sécrétion cumulative d'équivalents acides (µmol) ± erreur-type.

3.2. Les extraits induisent une dépression du contenu en ATP cytoplasmique

À l'instar du test de changement de pH, cet essai de mesure du contenu en ATP cytoplasmique a pour objectif de vérifier l'intensité, la rapidité et la durée du stress métabolique induit par les extraits de plantes au niveau cellulaire. Comme le test de changement de pH, il a également été utilisé dans les études portant sur la *Nigella sativa*

et la quercétine (voir articles en annexe). Les résultats sont exprimés selon le contenu en ATP cytosolique (% du témoin véhicule établi à 100%) en fonction du temps suite à chaque traitement et représentent la moyenne de trois ou quatre réplicas provenant de deux expériences différentes (n=2). Là encore, il n'a été possible d'effectuer une analyse statistique et de déterminer la signification, mais une interprétation a tout de même était faite (Martineau et al, 2009; voir en annexe).

Dans les cellules C2C12, tous les extraits, à l'exception de *Rhododendron groenlandicum*, montrent une réduction importante du contenu en ATP cytoplasmique après 1 heure de traitement (figure 6). Avec *Abies balsamea* et *Larix laricina* cette perte est maintenue sur une période de 6 heures, ce qui est en accord avec leurs effets observés sur le taux d'acidification du milieu extracellulaire. *Sorbus decora* est l'extrait qui a la moins longue durée d'action (1 heure). Après 6 heures d'incubation avec les autres extraits, leur effet a disparu, les valeurs d'ATP s'approchant de celle correspondant au témoin véhicule (DMSO). *Rhododendron groenlandicum* et *Sorbus decora* montrent une tendance à augmenter le contenu en ATP cellulaire en comparaison avec le témoin véhicule au terme du protocole expérimental.

Dans les cellules H4IIE, les effets des extraits sur le contenu en ATP semblent moins prononcés que dans les cellules C2C12, à l'exception de *Larix laricina*. Plusieurs extraits, de même que le contrôle positif FCCP montrent une augmentation du contenu en ATP cytoplasmique après 6 heures d'incubation.

Ces deux essais (pH et ATP) montrent clairement qu'il existe des différences dans les effets des extraits selon le type cellulaire étudié.

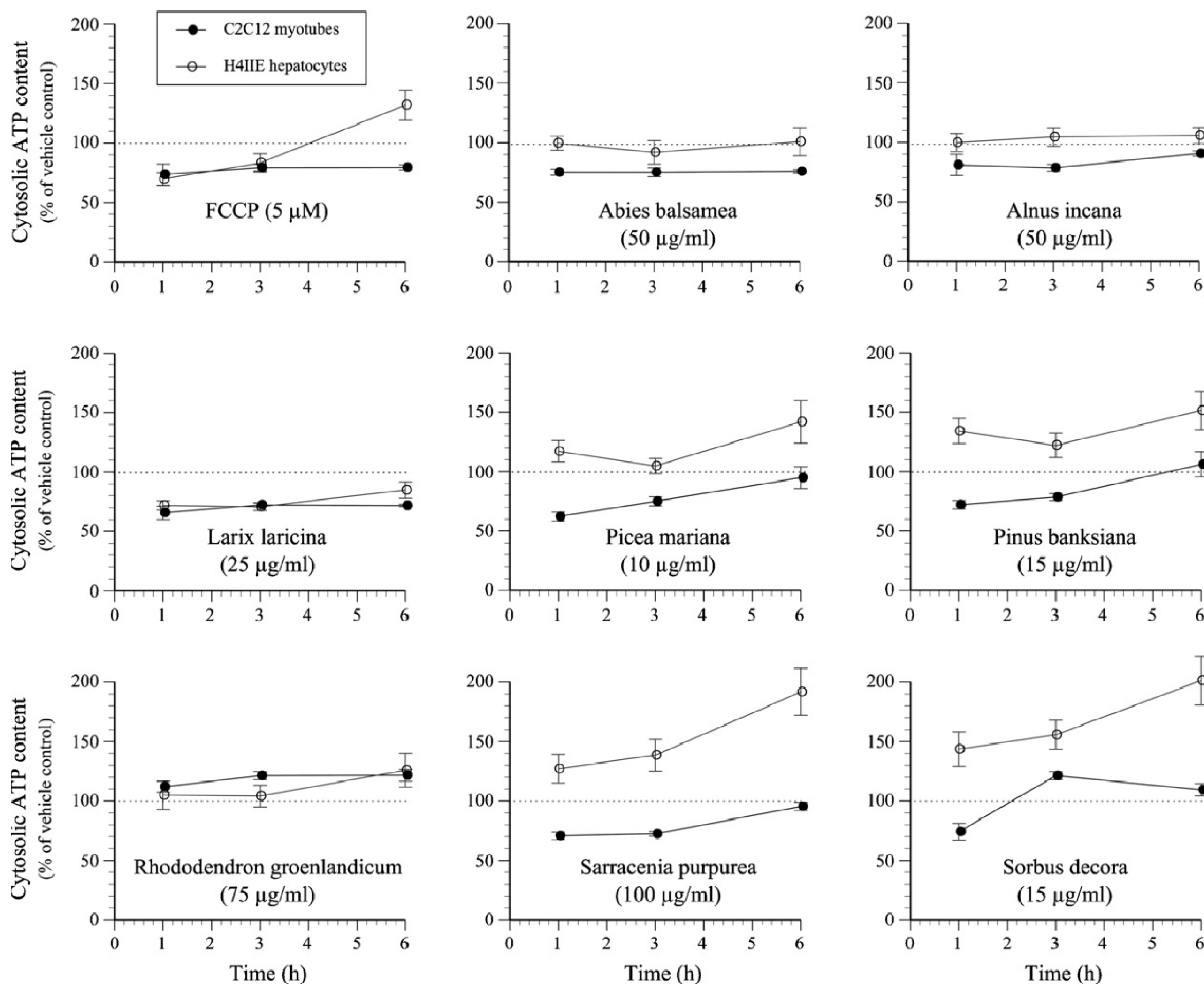

Figure 6. Effets des extraits sur le contenu en ATP cellulaire dans les cellules musculaires C2C12 (cercles fermés) et les hépatocytes H4IIE (cercles ouverts). Le découpleur FCCP est utilisé comme contrôle positif. Les résultats sont exprimés en pourcentage du témoin véhicule (moyennes de 3 ou 4 réplicas par condition et par temps ± erreur-type).

3.3. Détermination de la concentration maximale non-toxique des extraits dans les hépatocytes H4IIE

Le tableau 2 donne les concentrations maximales non-toxiques déterminées pour chaque extrait dans les cellules H4IIE (colonne de droite). Ces concentrations sont comparées à celles précédemment obtenues dans les cellules C2C12 (colonne du centre). Ces résultats montrent des différences dans la concentration maximale non-toxique de plusieurs extraits selon le type cellulaire.

Ainsi, *Abies balsamea*, *Larix laricinia*, et *Sorbus decora* ont une concentration maximale non-toxique plus élevé dans les hépatocytes que dans les cellules musculaires tandis que *Pinus banksiana*, *Rhododendron groenlandicum* et *Sarracenia Purpurea* ont une concentration maximale non-toxique plus élevé dans les C2C12 que dans les H4IIE. Ceci signifie que dans les tests de changement de pH et de mesure de l'ATP cytosolique, au niveau des hépatocytes, certains extraits ont été testés à une concentration toxique (supérieure à la concentration maximale tolérée par les cellules H4IIE) tandis que d'autres ont été testés à une concentration en deçà de la concentration maximale non-toxique supportée par les hépatocytes. Cela a surement grandement influé sur les résultats obtenus au niveau des cellules H4IIE et devrait être tenu en compte dans leur interprétation.

Tableau 2. Liste des extraits de plantes à l'étude ainsi que leurs concentrations maximales non-toxiques respectives déterminées dans chaque lignée cellulaire (C2C12 et H4IIE).

Extraits de plantes	Concentration maximale non-toxique dans les C2C12 (µg/ml)	Concentration maximale non-toxique dans les H4IIE (µg/ml)
Abies balsamea (**Abi**)	50	100
Alnus incana (**Aln**)	50	50
Larix laricina (**Lar**)	25	50
Picea mariana (**Pic**)	10	10
Pinus banksiana (**Pin**)	15	10
Rhododendron groenlandicum (**Rho**)	75	50
Sarracenia purpurea (**Sar**)	100	25
Sorbus decora (**Sor**)	15	60

3.4. Effets des extraits sur le contenu en triglycérides des hépatocytes traités au palmitate

Le traitement des cellules H4IIE avec du palmitate a pour objectif de mimer les conditions négatives (stéatose, inflammation, résistance à l'insuline) d'une diète grasse sur le foie. Il devient ainsi possible de vérifier les effets des extraits de plantes Cris sur un modèle cellulaire « pathologique ». Les cellules sont traitées 18 heures avec 150 µM palmitate en présence ou en absence des extraits à leurs concentrations maximales non-toxiques telles que déterminées dans les cellules H4IIE (voir tableau 2). Le médicament metformine (1 mM), également mis pour 18 heures en présence du palmitate et dont le mode d'action est similaire à celui des extraits (Martineau et al, 2009), sert de contrôle positif.

Le test AdipoRed permet de mesurer l'accumulation de triglycérides dans les cellules suite au traitement au palmitate et de vérifier si les extraits peuvent diminuer cette accumulation, ce qui pourrait se traduire par des effets bénéfiques dans une situation *in vivo* comme chez un patient diabétique. Comme le montre la figure 7, les cellules traitées au palmitate accumulent environ 3 fois plus de triglycérides que les cellules non traitées (BSA seulement). Curieusement, la metformine n'a montré aucune diminution du niveau de triglycérides dans les cellules traitées au palmitate alors que plusieurs études ont démontré le contraire (Lin et al, 2000; Puljak et al, 2008). Parmi les extraits, seul *Sorbus decora* a montré une diminution significative de l'accumulation de triglycérides dans les cellules. En effet, en présence de *Sorbus decora*, l'accumulation de triglycérides dans les cellules traitées au palmitate a été réduite de presque la moitié (1,5 fois au dessus du contrôle BSA comparativement à 3 fois).

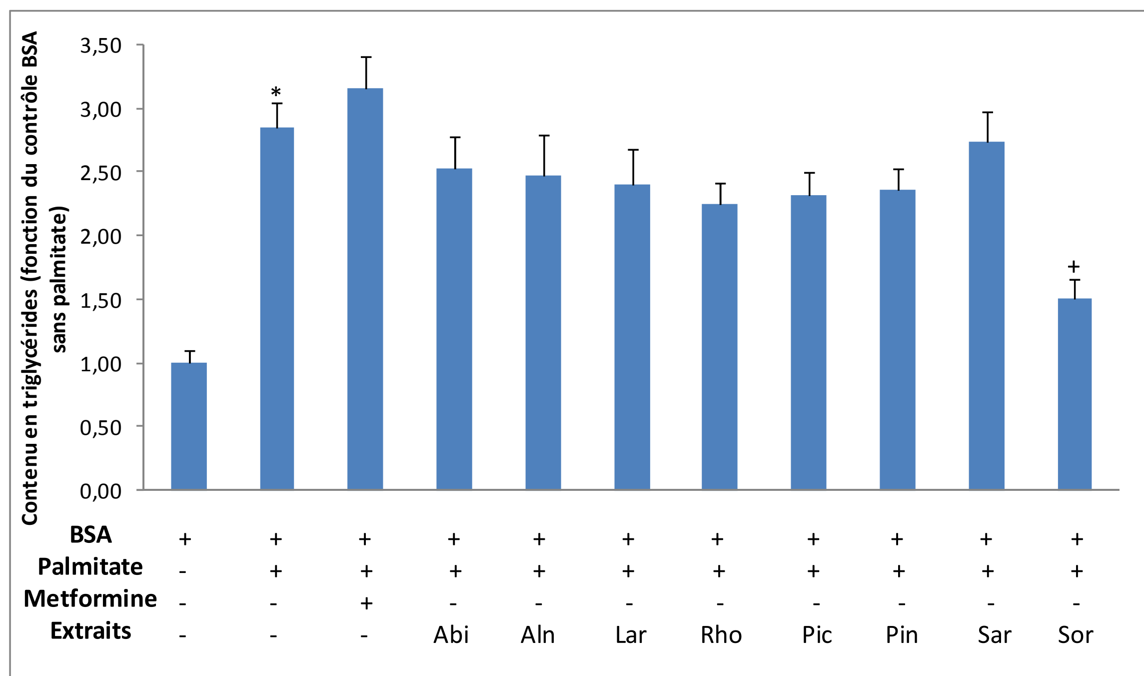

Figure 7. Effets des extraits sur le contenu en triglycérides dans des hépatocytes H4IIE traitées avec 150 µM palmitate pendant 18 heures. La metformine (1 mM) sert de contrôle positif. Les valeurs sont exprimées en fonction du témoin BSA sans palmitate et représentent la moyenne ± erreur-type de n=8 pour les conditions sans extraits et de n=4 pour les conditions avec extraits. Les extraits ont été utilisés à leur concentration maximale non-toxique dans les cellules H4IIE. La signification statistique des résultats a été obtenue par ANOVA à un facteur. * : significativement différent de BSA sans palmitate ($p < 0,0001$). + : significativement différent de BSA + Palmitate ($p < 0,0001$).

3.5. Effets des extraits sur la voie de l'insuline suite au traitement des cellules avec le palmitate

En parallèle avec le test AdipoRed, le niveau de phosphorylation de la protéine Akt, enzyme clé de la voie de signalisation de l'insuline, a été mesuré à l'aide d'un test ELISA. L'accumulation de triglycérides dans les cellules H4IIE suite à un traitement au palmitate altère la voie de signalisation de l'insuline et il est donc intéressant de vérifier si les différents extraits peuvent contrecarrer cette altération. Dans les cellules non-traitées au palmitate, l'ajout de 10 nM d'insuline pour 15 minutes augmente le niveau de phospho-Akt de 2,25 fois par rapport aux cellules témoins (figure 8A). En présence de 150 µM palmitate, l'ajout de l'insuline n'entraîne qu'une augmentation de 1,6 fois du niveau de phospho-Akt, ce qui est significativement moindre que l'augmentation obtenue

dans les cellules non-traitées au palmitate. Ces résultats démontrent que la voie de signalisation de l'insuline est affectée par le traitement au palmitate, la phosphorylation de l'Akt étant diminuée suite à une stimulation par l'insuline. En présence de metformine (1 mM), les cellules traitées au palmitate répondent mieux à une stimulation à l'insuline, le niveau de phosphos-Akt passant de 1,6 à 2,0 fois le contrôle BSA sans insuline. Ces résultats étaient attendus, car la metformine est connue pour améliorer la sensibilité à l'insuline *in vitro* et *in vivo* (Marchesini et al, 2001; Ginnarelli et al, 2003). Parmi les extraits, aucun n'entraîne une augmentation significative du niveau de phospho-Akt suite au traitement au palmitate. Curieusement, *Sorbus decora*, qui a montré une diminution importante du taux de triglycérides des cellules traitées au palmitate, n'a pas eu un effet d'amélioration de la signalisation à l'insuline (niveau de phospho-Akt à 1,6 fois le contrôle BSA sans insuline). Les niveaux d'Akt total ont également été mesurés afin de s'assurer que les effets obtenus pour l'Akt phosphorylé ne sont pas dus à un changement dans la quantité de cellules présentes dans les puits suite aux traitements. Comme le montre la figure 8B, les niveaux d'Akt sont les mêmes dans les différentes conditions expérimentales à l'exception du traitement avec *Picea mariana* qui a résulté en une augmentation significative de l'Akt total. Cette augmentation était inattendue et pourrait être due à une augmentation potentielle de la quantité de cellules suite aux traitements avec cet extrait. Toutefois, aucun dosage de protéines n'a été fait pour confirmer cette hypothèse.

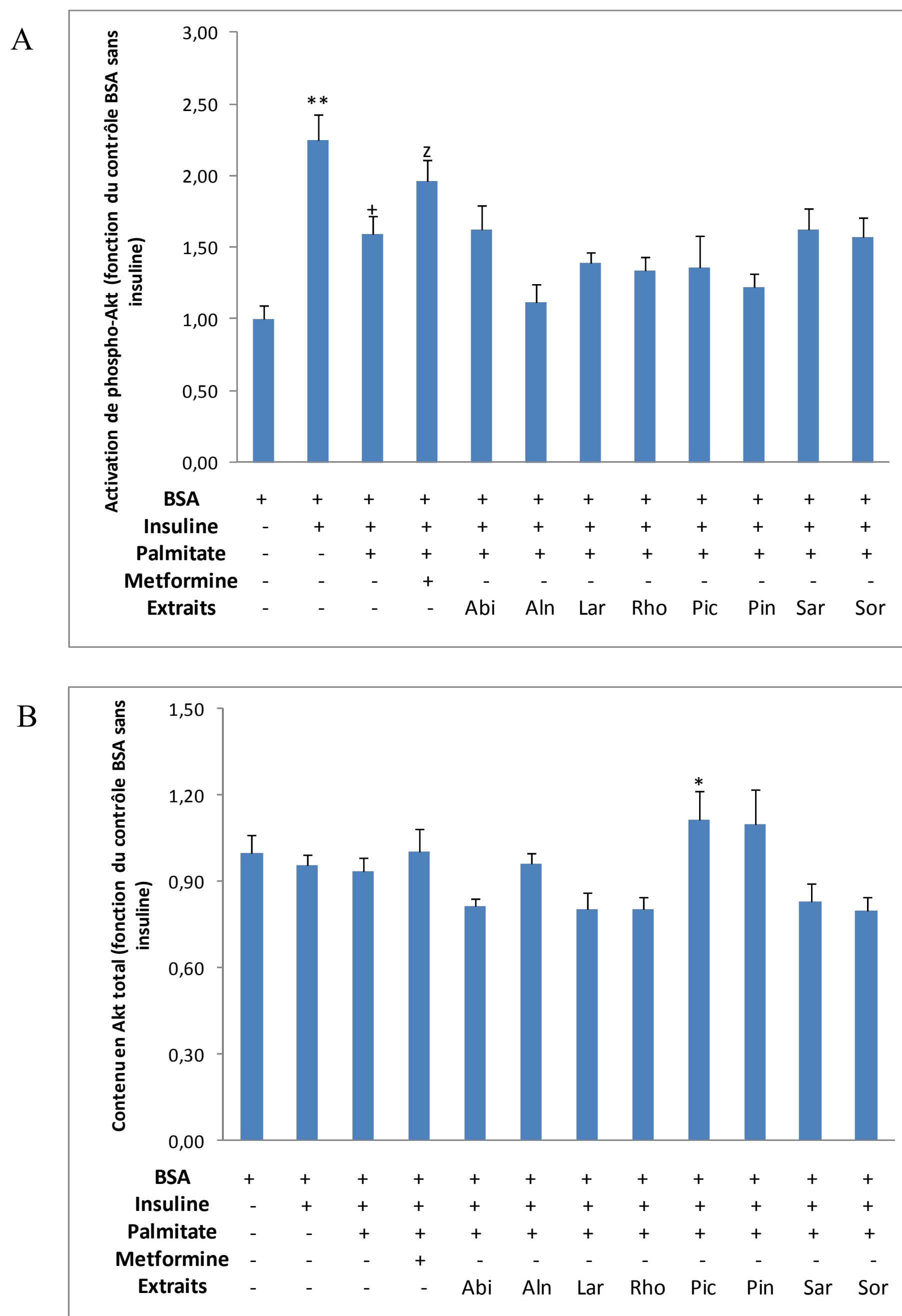

Figure 8. (A) Effets des extraits sur la phosphorylation d'Akt dans des hépatocytes H4IIE traitées avec 150 µM palmitate pendant 18 heures. La metformine (1 mM) sert de contrôle positif. Les valeurs sont exprimées en fonction du témoin BSA sans insuline et représentent la moyenne ± erreur-type de n=8 pour les conditions sans extraits et de n=4 pour les conditions avec extraits. L'insuline (10 nM) a été ajoutée à la fin des traitements pour une période de 15 minutes. Les extraits ont été utilisés à leur concentration maximale non-toxique dans les cellules H4IIE. La signification statistique des résultats a été obtenue par ANOVA à un facteur. ** : significativement différent de BSA sans insuline ($p < 0{,}0001$). + : significativement différent de BSA avec insuline ($p < 0{,}05$). z : significativement différent de BSA avec insuline et palmitate ($p < 0{,}05$). **(B)** Effets de ces mêmes traitements sur l'Akt total. Toutes les valeurs sont exprimées en fonction du témoin BSA sans insuline. * : significativement différent de BSA avec insuline et palmitate.

4. Discussion

La présente étude a comme premier objectif d'approfondir les connaissances déjà acquises sur les mécanismes d'action de huit plantes médicinales réputées antidiabétiques provenant de la pharmacopée Cris en mesurant leurs effets sur la chaîne respiratoire mitochondriale. Ces effets seraient responsables du développement d'un stress métabolique cellulaire, lui-même responsable de l'activation de l'enzyme AMPK et de l'augmentation conséquente de la prise de glucose par les cellules musculaires traitées avec les différents extraits (Spoor et al, 2006; Martineau et al, 2009). En utilisant des cellules musculaires C2C12 et des hépatocytes H4IIE, le flux de protons sécrétés par les cellules ainsi que le contenu en ATP cytosolique suite aux traitements avec les extraits de plantes ont été mesurés. Les résultats obtenus, dont la signification statistique n'a pu être établie, semblent montrer que les différents extraits agissent rapidement pour établir un stress métabolique (Martineau et al, 2009). La majorité des extraits augmente le taux d'acidification de façon importante dès les 20 premières minutes et leur effet s'amenuise par la suite. *Abies balsamea* et *Larix laricina* sont les deux extraits ayant le taux d'acidification le plus important et ils le maintiennent sur une période de 4 heures. La diminution du contenu en ATP cytosolique est plus importante après 1 heure et s'estompe par la suite. Là encore, les effets d'*Abies balsamea* et de *Larix laricina* sont les plus notables. Ces résultats sont en accord avec les effets de ces deux espèces sur la fonction respiratoire (Martineau et al, 2009). Toutefois, ces 2 extraits ne sont pas ceux qui ont le plus activé l'AMPK ni ceux qui ont le plus stimulé le transport de glucose dans les cellules musculaires (C2C12). *Sarracenia purpurea* et *Sorbus decora* ont eu des effets importants sur la prise de glucose et l'activation de l'AMPK, mais leurs effets ont été

plutôt limités dans les tests d'acidification du milieu extracellulaire et de mesure du contenu en ATP cytosolique (Spoor et al, 2006; Martineau et al, 2009). Ceci suggère qu'il n'existe pas forcément un lien direct entre l'importance du stress métabolique et l'activation de l'AMPK. Un faible stress de courte durée comme dans le cas d'un traitement avec *Sarracenia purpurea* ou *Sorbus decora* apparaît suffisant pour activer de façon optimale l'AMPK et permettre à la cellule d'augmenter sa prise de glucose. Un stress soutenu et important n'est pas forcément idéal pour la cellule, car ceci augmente la réduction du contenu en ATP et le recours à la glycolyse anaérobique. Ceci devrait ensuite augmenter la production et la sécrétion d'acide lactique, un des effets secondaires importants des médicaments de la classe des biguanides telle la metformine (Luft et al, 1978).

Ces essais ont également montré des différences dans les effets des extraits selon les types cellulaires. Ces différences avaient déjà été remarquées précédemment. L'essai sur la prise de glucose a également été fait sur des adipocytes 3T3-L1 et les résultats obtenus étaient différents de ceux obtenus avec les cellules C2C12 (Spoor et al, 2006). Ainsi, *Sorbus decora* n'a pas d'effets importants sur la prise de glucose par les cellules 3T3-L1 (contrairement a ce qui est noté dans les cellules C2C12) alors que *Abies balsamea* et *Rhododendron groenlandicum* ont des effets plus importants dans les cellules 3T3-L1 que dans les cellules C2C12. Les effets sur la prise de glucose et la phosphorylation d'AMPK, qui ont montré une bonne activité des extraits *Sarracenia purpurea* et *Sorbus decora*, ont eu lieu sur des cellules musculaires C2C12, tandis que les effets importants d'*Abies balsamea* sur la chaîne respiratoire ont été démontrés sur des mitochondries isolées de foie. Dans les essais sur le stress métabolique, *Picea mariana*,

par exemple, n'a que peu d'effet sur le taux d'acidification des cellules H4IIE, en accord avec ce qui a été observé au niveau des mitochondries isolées (Martineau et al, 2009). Le taux d'acidification a été plus important lorsque l'extrait a été ajouté aux cellules C2C12. *Abies balsamea* a des effets très importants sur le taux d'acidification du milieu extracellulaire des cellules H4IIE, dépassant même les effets du poison métabolique FCCP. Ainsi, il est possible que les effets d'*Abies balsamea* sur le métabolisme cellulaire soient plus importants au niveau du foie que du muscle et inversement dans le cas des extraits *Sarracenia purpurea* et *Sorbus decora*. Ceci peut être expliqué par des différences moléculaires au niveau de la chaîne de transport des électrons des mitochondries des cellules hépatiques et musculaires (Martineau et al, 2009). Ces différences entre les cellules hépatiques et musculaires suggèrent qu'il serait possible, voire souhaitable, de cibler spécifiquement certains tissus sachant que l'activation de l'AMPK n'a pas des effets bénéfiques à tous les niveaux, surtout chez un patient diabétique. Ainsi, l'activation de l'AMPK dans des cellules beta pancréatiques peut diminuer la sécrétion de l'insuline (da Silva Xavier et al, 2003) alors que dans des cellules de l'hypothalamus ceci entraîne l'activation d'un signal de faim (Kola, 2008).

Il est cependant important de noter que les concentrations testées dans les cellules H4IIE n'étaient pas optimales (les essais pour déterminer la concentration maximale non-toxique des extraits dans les hépatocytes n'ont été faits que par après). Ainsi, *Abies balsamea* et *Larix laricina* n'ont été testés qu'à la moitié de leur concentration maximale-non toxique dans les cellules H4IIE (50 µg/ml au lieu de 100 µg/ml pour *Abies balsamea* et 25 µg/ml au lieu de 50 µg/ml pour *Larix laricina*; Tableau 2). Ces deux extraits étant ceux qui ont eu le plus d'effets dans les tests de mesure de l'acidification du milieu

extracellulaire et du contenu en ATP cytosolique, il est probable que leurs effets auraient été encore plus importants (peut-être le double) s'ils avaient été testés à leur concentration maximale non-toxique. *Sorbus decora* a également été testé à une concentration bien en deçà de sa concentration maximale-non toxique dans les hépatocytes (15 µg/ml au lieu de 60 µg/ml). Il est possible que les effets de *Sorbus decora* soient plus importants dans les cellules H4IIE que ceux notés dans ces expériences. Ceci montrerait encore qu'il existe des différences dans les effets des extraits selon le tissu ciblé (effets de *Sorbus decora* plus importants dans les hépatocytes que dans les cellules musculaires). Aussi, quelques extraits, dont *Sarracenia purpurea*, ont été testés à des doses toxiques dans les cellules H4IIE. Ceci a surement affecté les résultats obtenus et il est alors difficile d'en tirer des conclusions solides. Une reprise de ces essais sur les hépatocytes avec les bonnes concentrations permettrait surement une interprétation plus juste des effets des espèces de plantes provenant de la pharmacopée Cris sur la chaîne respiratoire mitochondriale au niveau des cellules du foie.

La majorité des extraits testés dans cette étude a déjà démontré des résultats encourageants quant à leur capacité à augmenter la prise de glucose par les cellules musculaires (Spoor et al, 2006). Chez un patient diabétique, cela pourrait se traduire par une diminution de la glycémie et ainsi réduire les risques de complications dues à un haut taux de glucose sanguin. Toutefois, la grande majorité des personnes atteintes du diabète de type II souffre également d'obésité (Bokyo et al, 2000; Muoio et al, 2008). Chez une personne obèse, les hauts taux d'acides gras circulants sont un facteur de risque important de développer une résistance à l'insuline qui mènera éventuellement au diabète de type II. En effet, c'est l'accumulation de ces acides gras dans des tissus tels que le foie et le

muscle squelettique qui réduisent leur capacité à répondre à l'insuline. Ainsi, le fait de traiter des cellules, telles les hépatocytes H4IIE, avec un acide gras saturé (le palmitate) représente plus fidèlement la condition physiologique d'une personne résistante à l'insuline ou diabétique. Le second objectif de cette étude est donc de vérifier l'effet des extraits sur un modèle cellulaire hépatique mimant les conditions d'accumulation de triglycérides et de résistance à l'insuline propres à une personne diabétique souffrant de surpoids. Par un traitement avec les différents extraits de plantes cette étude vise plus spécifiquement à évaluer leur capacité à faire diminuer le niveau de triglycérides (test de l'AdipoRed) et à normaliser la signalisation du récepteur de l'insuline (mesure de la phosphorylation d'Akt). Le fait que les extraits ont démontré leur capacité à activer l'AMPK, qui favorise l'oxydation des graisses au niveau cellulaire, est encourageant quant à leur capacité à faire diminuer la quantité d'acides gras et autres composés issus de leur métabolisme qui sont grandement responsables du développement de la résistance à l'insuline.

La présence de palmitate à une concentration de 150 µM a presque triplé le taux de triglycérides présents dans les hépatocytes comparativement aux cellules n'ayant pas reçu l'acide gras (Figure 7). Cette forte accumulation de triglycérides s'est effectivement traduite par une diminution de la capacité des cellules à répondre à l'insuline comme le démontre la réduction du taux de phosphorylation de l'Akt (Figure 8A). Parmi les extraits, seul *Sorbus decora* a diminué significativement (de moitié) le niveau de triglycérides des cellules traitées au palmitate. *Sorbus decora* est aussi un des extraits ayant le plus activé l'AMPK. Toutefois, ni la metformine, ni *Sarracenia purpurea* (autre extrait ayant activé fortement l'AMPK), n'ont diminué significativement le niveau de

triglycérides. Dans le cas de la metformine, ceci surprend un peu car, sachant qu'elle agit via l'activation de l'AMPK et que cette activation entraîne, entre autres, une oxydation des graisses, une diminution significative du taux de triglycérides était attendue. Des études ont également démontré que la metformine prévient l'accumulation de lipides intracellulaire et diminue le taux de gras dans le foie de souris et personnes obèses (Lin et al, 2000; Bugianesi et al, 2005). L'insuccès de la metformine à faire diminuer le taux de triglycérides dans ce modèle peut être expliqué soit par la concentration utilisée (1 mM) soit par la durée du traitement (18 heures). Toutefois, cette même concentration et une durée de traitement similaire (16 heures) ont démontré des effets positifs de la metformine sur la diminution du taux de triglycérides dans un modèle cellulaire hépatique (cellules HTC) utilisée dans une autre étude (Puljak et al, 2008). Également, la metformine à 1 mM pour 18 heures a eu des effets positifs sur la sensibilisation à l'insuline des cellules H4IIE traitées au palmitate, suggérant que la dose utilisée et la durée du traitement ne sont pas nécessairement à remettre en cause. De façon intéressante, une étude comparative des effets de la metformine et de la rosiglitazone chez des patients atteints du diabète de type II a montré que la metformine améliore la sensibilité hépatique à l'insuline sans modifier le taux de gras dans le foie (Tiikkainen et al, 2004). Ceci est plus en accord avec ce qui a été observé dans la présente étude.

Les résultats obtenus dans ces expériences sur les effets de la metformine sur le taux de triglycérides intracellulaire pourraient aussi être expliqué par les limites des modèles cellulaires. Déjà, les résultats divergent entre deux modèles cellulaires hépatiques, entre cellules H4IIE et cellules HTC (qui sont une lignée cellulaire dérivant d'un hépatome de rat). Il devient donc difficile d'extrapoler les résultats à la condition *in*

vivo où la metformine n’est pas directement mis en contact avec les cellules mais passe par le système digestif (digestion, absorption, métabolisme…) et où il existe de nombreuses interactions entre les différents systèmes biologiques. Le fait que la metformine n’ait pu faire diminuer le taux de triglycérides dans ce modèle ne signifie pas nécessairement son incapacité à le faire *in vivo*.

En ce qui concerne les extraits, les deux ayant montré la meilleure activation d’AMPK, *Sarracenia purpurea* et *Sorbus decora*, n’ont pas eu les mêmes effets sur le taux de triglycérides des hépatocytes traitées au palmitate. *Sarracenia purpurea* n’a eu que très peu d’effets en comparaison avec *Sorbus decora*, alors que leur activation d’AMPK sur 18 heures est assez comparable (Martineau et al, 2009). Il faut noter, toutefois, que la mesure de l’activation d’AMPK par les extraits a eu lieu sur des cellules musculaires C2C12, alors que la mesure du taux de triglycérides et de la sensibilité à l’insuline s’est effectuée sur des hépatocytes H4IIE. Comme les effets des extraits paraissent différents selon les tissus étudiés, il est concevable que les effets sur les cellules C2C12 et les cellules H4IIE diffèrent. Il se peut donc aussi que l’activation d’AMPK par les extraits varie selon qu’ils agissent sur le muscle squelettique ou le foie. Pour cela, il aurait été intéressant de mesurer l’activation d’AMPK induite par les extraits au niveau des cellules H4IIE en parallèle aux expériences visant à mesurer les effets des extraits sur des hépatocytes traités au palmitate. Les études futures devraient s’attarder à cette considération.

Les résultats obtenus sur la phosphorylation de l’Akt montrent qu’aucun des extraits n’a réussi à contrecarrer les effets négatifs induits par le palmitate (Figure 8A). Même *Sorbus decora*, si efficace à faire diminuer la quantité de gras dans les cellules, n’a

pu augmenter le taux de phosphorylation de l'Akt lorsque mis en présence du palmitate. Par contre, la metformine a atténué partiellement les effets du palmitate sur la voie de l'insuline en augmentant le taux de phosphorylation de l'Akt. Ceci est en accord avec les effets connus de la metformine sur l'amélioration de la sensibilité à l'insuline, malgré le fait que le taux de triglycérides intracellulaire n'ait pas diminué et concorde également avec l'étude mentionnée plus haut montrant une amélioration de la sensibilité à l'insuline par la metformine sans changement du degré de stéatose hépatique (Tiikkainen et al, 2004).

Il existerait un lien direct entre l'AMPK et la voie de signalisation de l'insuline (Tao et al, 2010). mTOR (*mammalian target of rapamycin*), une protéine kinase, est un important régulateur de l'action de l'insuline. En présence d'insuline et d'une quantité suffisante de glucose, mTOR est activé et ceci entraîne la phosphorylation en serine/threonine d'IRS1 et donc une atténuation du signal à l'insuline (Um et al, 2006; Yang et al, 2007). L'activation d'AMPK entraînerait l'inhibition de mTOR (Tao et al, 2010) et serait donc bénéfique pour la signalisation de la voie de l'insuline. Ceci pourrait expliquer les effets de la metformine qui aurait agit directement sur la voie de signalisation de l'insuline (en favorisant l'inhibition de mTOR) et augmenté le taux de phosphorylation de l'Akt sans pour autant avoir pu faire diminuer significativement le taux de triglycérides.

Il reste par contre difficile d'expliquer l'absence d'effets des extraits. Comme dans le cas des effets de la metformine sur le taux de triglycérides, ceci pourrait être expliqué par les limites des modèles *in vitro*, qui ne permettraient pas de détecter une amélioration de la sensibilité à l'insuline comparativement à un modèle *in vivo*. *Sorbus*

decora a réussi à diminuer le taux de triglycérides sans influer sur la voie de l'insuline (à l'inverse de la metformine). Ceci montre qu'il n'existe pas forcement un lien direct entre l'augmentation de l'oxydation des graisses hépatiques (inférée selon la diminution de l'accumulation de triglycérides) et le rétablissement de la sensibilité à l'insuline des hépatocytes insulino-résistants. Également, la présence continue du palmitate et la concentration utilisée pourrait contrecarrer les effets des extraits si ceux-ci sont limités dans le temps. Toutefois, retirer le palmitate avant le traitement avec les extraits n'a pas donné de résultats encourageants car, les effets du palmitate se dissipent rapidement après le retrait de celui-ci (essai fait en laboratoire, résultats non-publiés). Une autre possibilité serait que la période de temps durant laquelle les extraits ont été ajoutés n'était pas optimale. Il conviendrait donc de tester à l'avenir l'ajout des extraits à différents temps tout le long du traitement des cellules H4IIE au palmitate. À titre d'exemple, la metformine et les extraits pourraient être ajoutés aux cellules toutes les 6 heures sur une période de 18 heures, tout en maintenant le palmitate à la même concentration. Ceci mimerait mieux le mode de vie d'une personne antidiabétique qui prend normalement ses médicaments (ou les extraits) plusieurs fois par jour. Le fait qu'un des extraits, *Sorbus decora*, ait aussi significativement diminué le taux de triglycérides dans des cellules hépatiques H4IIE traitées au palmitate reste quand même très encourageant quant à la capacité de cet extrait à faire diminuer la quantité de gras dans le foie d'une personne obèse. Ceci pourrait se traduire par un effet antidiabétique (ou de résistance à l'insuline), même ci *Sorbus decora* n'a pas permis d'améliorer la sensibilité à l'insuline des cellules H4IIE rendues résistantes à l'insuline dans le cadre de ce projet. D'ailleurs, des études *in vivo* menées avec *Sorbus decora* au laboratoire ont démontrées le potentiel

hypoglycémiant et de sensibilisation à l'insuline de cette espèce dans divers modèles de rats ou de souris diabétiques ou résistants à l'insuline (Vianna et al, 2009). Ces différents modèles n'étaient toutefois pas devenus diabétiques ou résistants à l'insuline suite à une diète riche en gras, mais plutôt de façon génétique (rats STZ, souris KK-A^{y}) ou suite à la consommation d'eau enrichi de 10% de glucose. Des études ultérieures devront détailler davantage les mécanismes d'action des plantes afin de mieux comprendre leurs subtilités d'action qui se traduisent par un effet bénéfique *in vivo*.

5. Conclusion générale

En conclusion, cette étude suggère que les extraits induisent un stress métabolique en affectant la chaîne respiratoire mitochondriale. C'est ce stress métabolique qui est fort probablement responsable de l'activation de l'AMPK et des effets antidiabétiques observés lorsque ces extraits sont mis en présence de cultures cellulaires (augmentation de la prise basale de glucose par les cellules musculaires C2C12). Généralement, ce stress métabolique est de nature transitoire, tout comme la chute d'ATP intracellulaire qu'elle entraîne. Ceci est encourageant et suggère que les plantes Cris puissent optimiser les effets bénéfiques d'un tel stress tout en réduisant les risques de toxicité chez un patient diabétique, notamment par acidose métabolique. L'étude montre également des différences au niveau des effets des extraits quant au type cellulaire et que l'intensité du stress métabolique ne se répercute pas directement sur l'activation de l'AMPK. Malgré le fait que la majorité des extraits ont activé l'AMPK dans des cellules C2C12, ceci ne s'est pas traduit par une diminution du taux de triglycérides (à l'exception notable de *Sorbus decora*) ni par une amélioration de la sensibilité à l'insuline dans une modèle cellulaire hépatique « pathologique » de cellules H4IIE traitées au palmitate. Ceci peut être dû au fait que les effets des extraits sur l'activation de l'AMPK peuvent être différents dans des cellules H4IIE comparativement aux cellules C2C12 et que donc, il serait pertinent de mesurer l'activation de l'AMPK dans des cellules H4IIE suite au traitement avec les extraits. D'autres tests (*in vitro* et *in vivo*) visant à déterminer et à mieux comprendre les effets antidiabétiques de ces extraits de plantes provenant de la pharmacopée Cris sont actuellement en cours au laboratoire ainsi que dans les différents labos des différents membres de l'équipe travaillant sur ce projet. Il est ainsi espéré que les résultats obtenus

permettront d’améliorer les connaissances sur les différents extraits et ainsi améliorer l’élaboration de traitements antidiabétiques à destination des populations autochtones.

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Enhancement of muscle cell glucose uptake by medicinal plant species of Canada's native populations is mediated by a common, Metformin-like mechanism

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ABSTRACT

Aim: The purpose of the present study was to elucidate the mechanisms of action mediating enhancement of basal glucose uptake in skeletal muscle cells by seven medicinal plant products recently identified from the pharmacopeia of native Canadian populations (Spoor et al., 2006).
Methods: Activity of the major signaling pathways that regulate glucose uptake was assessed by western immunoblot in C2C12 muscle cells treated with extracts from these plant species. Effects of extracts on mitochondrial function were assessed by respirometry in isolated rat liver mitochondria. Metabolic stress induced by extracts was assessed by measuring ATP concentration and rate of cell medium acidification in C2C12 myotubes and H4IIE hepatocytes. Extracts were applied at a dose of 15–100 μg/ml.
Results: The effect of all seven products was achieved through a common mechanism mediated not by the insulin signaling pathway but rather by the AMP-activated protein kinase (AMPK) pathway in response to the disruption of mitochondrial function and ensuing metabolic stress. Disruption of mitochondrial function occurred in the form of uncoupling of oxidative phosphorylation and/or inhibition of ATPsynthase. Activity of the AMPK pathway, in some instances comparable to that stimulated by 4 mM of the AMP-mimetic AICAR, was in several cases sustained for at least 18 h post-treatment. Duration of metabolic stress, however, was in most cases in the order of 1 h.
Conclusions: The mechanism common to the seven products studied here is analogous to that of the antidiabetic drug Metformin. Of interest is the observation that metabolic stress need not be sustained in order to induce important adaptive responses. The results support the use of these products as culturally adapted treatments for insulin resistance and hyperglycemia in susceptible aboriginal populations where adherence to modern diabetes pharmaceuticals is an issue. The mechanism reported here may be widespread and mediate the antidiabetic activity of traditional remedies from various other cultures.
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1. Introduction

Aboriginal populations the world over are predisposed to the development of type II diabetes (Yu and Zinman, 2007). When this susceptibility is compounded by the cultural disconnection of modern pharmaceuticals, the prevalence rates of diabetic complications can reach alarming levels. These complications more than diabetes itself impact quality of life and longevity and represent important social and economic burdens. In native populations of Canada, such as the Cree of Iiyiyiu Istchii (James Bay region), the prevalence rate of type II diabetes is 3–5 higher than in non-native populations (Kuzmina and Dannenbaum, 2004; Kuzmina et al., 2008; Young et al., 2000) and, due to cultural disconnection to modern treatment options, the prevalence rate of complications is disproportionately high. Our team has taken a novel approach to address this specific situation; in close collaboration with the Cree of Iiyiyiu Istchii, we are attempting to improve diabetes treatments by developing culturally adapted complementary and alternative options that are based on products of this population's own pharmacopeia.

Plant products with hyperglycemia-normalizing activity are common in the traditional medicine of cultures throughout the world (Haddad et al., 2006; Marles and Farnsworth, 1995). The efficacy of several of these products has been demonstrated in clinical

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and animal studies. In some cases, active principles have also been identified. These natural products represent a rich source of alternatives and complements to the limited number of antidiabetic medications currently on the market. Furthermore, in populations with limited access to modern pharmaceuticals or with a cultural preference for traditional remedies, these products often represent the main treatment option for diabetes.

Perhaps the most important testimony to the importance and efficacy of antidiabetic natural products comes from the widely used antidiabetic drug Metformin and the other members of the biguanide class. Indeed, these drugs are based on naturally occurring guanidines isolated in the 1920s from *Galega officinalis* (French lilac), a plant used for centuries to treat diabetes (Cavaliere, 2007; Witters, 2001). The biguanides act not by increasing insulin secretion, but rather by decreasing gluconeogenesis and increasing glucose uptake (Giannarelli et al., 2003). While the biguanides have been on the market since the 1950s, the major breakthrough in understanding their mode of action occurred in 2001 when the enzyme adenosine-monophosphate-activated protein kinase (AMPK) was found to play a central role in their effects (Zhou et al., 2001). This highly conserved enzyme is exquisitely sensitive to disruptions in cellular energy homeostasis and is now recognized as both a master regulatory enzyme of metabolism and an important therapeutic target for diabetes and metabolic diseases (Misra, 2008; Viollet et al., 2009). Activation of AMPK triggers cytoprotective responses to acutely restore energy homeostasis and to chronically protect against future perturbations, including stimulation of transport and oxidation of glucose and fats, increased expression of key genes of glucose and fat metabolism, and mitochondrial biogenesis (Reznick and Shulman, 2006; Winder, 2001).

In recent years, many plant products and naturally occurring compounds have been shown to activate AMPK (Ahn et al., 2008; Collins et al., 2007; Hayashi et al., 2000; Hwang et al., 2005; Lee et al., 2007, 2006; Liu et al., 2007; Mooney et al., 2008; Park et al., 2007; Zang et al., 2006). In many, if not all cases, a site of action of these products is the mitochondrial inner membrane or a protein thereof (Dorta et al., 2005; Lee et al., 2006; Polya, 2003; Trumbeckaite et al., 2006), with the effect of disrupting oxidative phosphorylation and decreasing the capacity for aerobic ATP synthesis. If mitochondrial energy production becomes insufficient to meet the cellular energy demand, energy homeostasis is perturbed and AMPK is activated. Targeting of energy transduction pathways is consistent with the defensive antimicrobial and insecticidal functions of plant secondary metabolites (Polya, 2003). Mild and transient disruption of mitochondrial function is believed to be the primary mechanism of action of Metformin, shown to inhibit oxidative phosphorylation at complex I of the electron transport chain (El-Mir et al., 2000; Owen et al., 2000). As aerobic energy production is compromised, the cell must increasingly rely on ATP synthesized anaerobically through glycolysis. Consequently, the primary danger associated with this mode of action is metabolic acidosis. Indeed, acidosis is the most important complication of biguanide use (Luft et al., 1978). Furthermore, consumption of *Galega officinalis* by grazing livestock can result in this condition as well (Cavaliere, 2007).

Our team has recently conducted a successful screening project of medicinal plant species of the Canadian boreal forest stemming from the traditional medicine of the Cree of Iiyiyiu Istchii (Leduc et al., 2006; Spoor et al., 2006). Seven of eight ethnobotanically selected species were found to enhance glucose uptake in skeletal muscle cells in the absence of insulin by 10–45% (Spoor et al., 2006), an effect similar to that of Metformin (Klip et al., 1992; Spoor et al., 2006). This activity had not previously been ascribed to these species. The goal of the present work was to determine the molecular events through which these effects are mediated. Our findings indicate that all seven plant products act through the AMPK pathway and that activation of this pathway results from a transient disruption of mitochondrial function. These findings further support the use of these products as culturally adapted alternative anti-hyperglycemic therapies. These findings also suggest that this mode of action may be widespread among medicinal plant species and natural health products with antidiabetic activity.

2. Materials and methods

2.1. Plant materials

The following eight products of Canadian boreal forest medicinal plant species were the objects of this study: inner bark of *Abies balsamea* (L.) Mill.; inner bark of *Alnus incana* ssp. *Rugosa* (Du Roi) R.T. Clausen; inner bark of *Larix laricina* K. Koch; cones of *Picea mariana*; cones of *Pinus banksiana* Lamb.; leaves of *Rhododendron groenlandicum* (Oeder) Kron & Judd; leaves of *Sarracenia purpurea* L.; inner bark of *Sorbus decora* C.K. Schneid. The collection, handling, and extraction of plant material have previously been described (Spoor et al., 2006). Briefly, plant material was collected in 2003 in Mistissini, QC, Canada. Material was collected from several specimens and pooled. Species were identified by a plant taxonomist (Dr. Alain Cuerrier, Montreal Botanical Garden, Montreal, QC) and voucher specimens were deposited at the Marie-Victorin Herbarium of the Montreal Botanical Garden (specimens # Mis03-1, Mis03-2, Mis03-4, Mis03-5, Mis03-8, Mis03-12, Mis03-14, and Mis03-15). Plant material was extracted at the University of Ottawa. Material from each species was dried in a fruit dryer at 35 °C, cleaned, and ground using a Wiley Mill with a mesh size of 2 mm. Ground material was extracted twice for 24 h in 80% ethanol (10 ml/g dry material) on a mechanical shaker at ambient temperature and then filtered through Whatman #1 paper. The supernatants of first and second extractions were pooled, evaporated to dryness in a rotary evaporator (RE500; Yamamoto Scientific Co., Ltd., Tokyo, Japan) with a water bath temperature of 40 °C, and lyophilized in a freeze dryer (Super Moduylo; Thermo Fisher, Ottawa, ON) to produce a crude ethanol extract (referred to as crude extract from this point on). Extraction yields (%) were as follows: *Abies balsamea* 15; *Alnus incana* 26; *Larix laricina* 24; *Picea mariana* 21; *Pinus banksiana* 9; *Rhododendron groenlandicum* 31; *Sarracenia purpurea* 25; *Sorbus decora* 9 (Spoor et al., 2006). Dried crude extracts were preserved at 4 °C in a dessicator and protected from light until further use. Extracts were solubilized in dimethyl sulfoxide (DMSO; Sigma–Aldrich, Oakville, ON) at 1000× their final working concentration, filter sterilized, aliquoted, and stored at −20 °C. On the day of an experiment, an aliquot was thawed and diluted in culture medium at 1:1000. Final working concentrations in microgram crude extract/ml were as follows: *Abies balsamea* 50; *Alnus incana* 50; *Larix laricina* 25; *Picea mariana* 10; *Pinus banksiana* 15; *Rhododendron groenlandicum* 75; *Sarracenia purpurea* 100; *Sorbus decora* 15. These concentrations have previously been determined to be optimal and non-toxic for C2C12 muscle cells over a treatment duration of 24 h (Spoor et al., 2006). Final DMSO concentration was 0.1% in all experiments. A preliminary phytochemical characterization of the extracts, including determination of total concentration of phenolics and identification and quantification of marker compounds, has been reported elsewhere (Spoor et al., 2006).

2.2. Cell culture

C2C12 murine skeletal myoblasts and H4IIE rat hepatocytes were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured at 37 °C in a humidified 5% CO_2/95% air atmosphere. Culture reagents were purchased from Wisent (St-Bruno, QC). As previously described (Benhaddou-Andaloussi et al., 2008; Martineau et al., 2006; Spoor et al., 2006; Vuong

et al., 2007), myoblasts were proliferated to confluence in high-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS; Sigma–Aldrich), and antibiotics. These cells were then differentiated into multinucleated myotubes over a 7-day period in high-glucose DMEM supplemented with 2% HS. Experiments with C2C12 cells were always timed to end on day 7 of differentiation. Hepatocytes were proliferated to confluence in high-glucose DMEM supplemented with 10% FBS and antibiotics. H4IIE experiments were performed between post-confluence days 1 and 3.

2.3. Western immunoblot studies of phospho-Akt, -AMPK, and -ACC contents

Content of phosphorylated protein kinase Akt (Ser 473) was measured by western immunoblot in order to assess activation of the insulin signaling pathway. Similarly, contents of phosphorylated AMPKalpha (catalytic subunit; Thr 172) and phosphorylated acetyl-coA carboxylase (ACC; Ser 79) were measured as markers of activation of the AMPK pathway. Reagents were purchased from Sigma–Aldrich unless otherwise noted. Primary antibodies were purchased from Cell Signaling Technologies Inc. (cat. # 9271, 2531, 3661; Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories Inc. (cat. # 111-035-144; West Grove, PA). C2C12 cells were seeded in 6-well plates and proliferated and differentiated as above. Cells were treated with extract or vehicle (DMSO) 18, 6, or 1 h before lysis on day 7 of differentiation. 5-Aminoimidazole-4-carboxamide-1β-riboside (AICAR; Toronto Research Chemicals Inc., North York, ON) was used as a positive control for the AMPK pathway; AICAR was dissolved in water and applied at a final concentration of 4 mM to a subgroup of vehicle control wells 30 min prior to lysis. At the end of the treatment period, plates were placed on ice and cells were rinsed twice with ice-cold phosphate-buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 2.68 mM KCl, 0.137 M NaCl) and covered with 250 μl/well of HEPES lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, 5% glycerol, 1% Triton X-100, 1% Na deoxycholate, 0.1% Na dodecyl sulphate (SDS)) containing a cocktail of protease inhibitors (Complete-Mini EDTA-free; Roche Diagnostics, Laval, QC; supplemented with 1 mM phenylmethylsulphonyl fluoride) and phosphatase inhibitors (10 mM NaF, 100 μM Na_3VO_4, 1 mM $Na_4P_2O_7$). Cells were scraped and transferred to microcentrifuge tubes. The tubes were vortexed and kept on ice for 30 min with frequent vortexing. Tubes were then centrifuged at 600 × *g* for 10 min at 4 °C. The supernatants were decanted into new tubes, and these lysates were frozen at −80 °C until analysis. The protein content of the cell lysates was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. An equal amount of total protein from each sample was denatured by boiling 5 min in reducing sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 1% SDS) (Laemmli, 1970). One hundred micrograms of each sample in a 100 μl volume were resolved by SDS-polyacrylamide gel electrophoresis (Shapiro et al., 1967) using a Protean IIxi apparatus (Bio-Rad Laboratories, Hercules, CA). The resolving gel was composed of a 6.5% acrylamide phase over a 10% acrylamide phase, and the stacking gel was 5% acrylamide. Electrophoresis was performed at 4 °C in migration buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS) at 50 mA for 3 h followed by 25 mA for 14 h. Resolved samples were then electrotransferred (Towbin et al., 1979) to Immobilon-P polyvinylidene fluoride membrane (Millipore Corp., Billerica, MA) using a Trans-Blot cell (Bio-Rad Laboratories) in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 10% methanol, 0.02% SDS) at 4 °C, 900 mA, for 1.5 h. Membranes were stained with Ponceau Red to confirm equal loading, then blocked for 1.5 h in 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline (TBS) plus Triton X-100 (TBST; 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100). Blocked membranes were incubated overnight at 4 °C with constant agitation in primary antibody solution (antibody at 1:1000 in TBST plus 1% BSA and 0.5% NaN_3). Membranes were rinsed in TBST and incubated 1.5 h at ambient temperature in secondary antibody solution (antibody at 1:100 000 in TBST plus 0.5% BSA). Membranes were then thoroughly washed in TBST and TBS, and treated for 1 min with ECL reagent (Amersham/GE Healthcare, Baie d'Urfé, QC). Membranes were exposed to blue-light sensitive ECL film (Amersham/GE Healthcare) for the appropriate duration for maximal signal without film saturation. Films were developed manually using D-19 developer and RapidFixer (Eastman Kodak Co., Rochester, NY). Developed films were scanned using a Hewlett Packard 6100 flatbed scanner (HP; Palo Alto, CA) with HP DeskScan II software. Densitometry analysis was then performed using Image 1.63 software (National Institutes of Health, Bethesda, MD). Three replicates, each from a different cell passage, were performed for each condition. For each series of replicates, all samples were simultaneously subjected to electrophoresis and transferred to a single membrane. Data from the densitometric analysis of each replicate series were normalized to the vehicle control of that series. Normalized data from the three series were then pooled.

2.4. Mitochondrial respiration studies

The effects of plant extracts on oxygen consumption of isolated mitochondria were assessed by oxygraphy with a Clark-type oxygen microelectrode system. Mitochondria were isolated from the liver of male Wistar rats weighing between 200 and 225 g. 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. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and underwent laparotomy. 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, 250 mM NaCl, 4.8 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgSO_4$) at 22 °C prior to excision. Mitochondria were isolated from 2 g of liver by the method of Johnson and Lardy (Johnson and Lardy, 1967). Briefly, tissue was homogenized on ice using a Teflon potter homogenizer in ice-cold Tris-sucrose 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. The supernatant was centrifuged at 15 000 × *g* for 5 min at 4 °C. The pellet from this centrifugation was delicately washed once in the same buffer, centrifuged, washed once in EGTA-free buffer, and centrifuged again at 15 000 × *g*. The final pellet, containing viable mitochondria, was suspended in EGTA-free homogenizing buffer and kept on ice. Protein content of the homogenate was determined by Lowry's protein assay (Lowry et al., 1951). O_2 consumption was measured at 25 °C in a Hansatech Oxygraph apparatus (Norfolk, UK) with a 1 ml reaction chamber, as previously described (Ligeret et al., 2008; Morin et al., 2001). 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 mg mitochondrial protein (RBOC or state 4 respiration) was determined. One microliter 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 phosphoryla-

tion (state 3 respiration) was induced by the addition of 200 μM (final concentration) ADP and the rate of ADP-stimulated O_2 consumption per mg 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: (1) the increase in the RBOC (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 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 mg protein of the vehicle control experiments for a given session was calculated by subtracting the average RBOC from the average RASOC. For (1) 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 (2) above, the FC measured in a given experiment was expressed as a percentage of the average control FC for the session to give the % residual FC. The compound 2,4-dinitrophenol (Sigma–Aldrich) was used at 5 μM as a reference uncoupler, whereas oligomycin A (Sigma–Aldrich) was used at 0.5 μM as a reference ATPsynthase inhibitor.

2.5. Rate of acidification of extracellular medium

A spectrophotometric assay of change in cell culture medium pH over time, quantitative between pH 7.2 and 6.4, was developed based on similar assays (Schornack and Gillies, 2003; Yang and Balcarcel, 2003). The assay medium consisted of Dulbecco's PBS containing Phenol Red as a pH indicator and modified to contain a total of 2 mM phosphate (for approx. 20% of the buffering capacity of Dulbecco's original recipe) while keeping other ion concentrations within the physiological range of extracellular fluid. The composition of this modified Dulbecco's PBS (mD-PBS) was: 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 (Sigma–Aldrich Phenol Red 0.5% solution), and deionized ultra-filtered water (Fisher Scientific, Ottawa, ON). This formulation resulted in a pH of 7.1, which was adjusted to 7.20 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 Inc., Montreal, QC) was measured at ambient temperature at 530 and 450 nm using a Wallac Victor 2 plate reader (Perkin-Elmer, St-Laurent, QC) and the ratio of abs 530/abs 450 was calculated. The relationship between pH and the log of this ratio was observed to be linear over the range of pH of 6.4–7.4, in agreement with a pKa of 6.9 for balanced-salt phosphate buffers. The following function was used to model the relationship between pH and absorbance over the pH range of 6.4–7.2: $\text{pH} = 0.765 \times \ln(\text{abs } 530/\text{abs } 450) + 7.61$ ($R^2 = 0.99$). A titration experiment was performed to determine the buffering capacity of mD-PBS. This capacity was observed to be nearly linear between pH 6.2 and 7.2 and was calculated to be 1.075 mM equivalents per pH units between 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 a 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. Because DMSO was observed to stimulate acidification, as noted by others (Gerber et al., 1996), extracts were solubilized in 80% ethanol at 1000× their final concentration, for a final ethanol concentration of 0.08%. The addition of extracts or controls affected the pH of mD-PBS and therefore all treatments were adjusted to pH 7.2 separately immediately prior to the assay. 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.

2.6. Cytosolic ATP assays

Total cytosolic ATP was measured in cell lysates by luminescence using the ATPlite assay kit (Perkin-Elmer, Waltham, MA), 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. Cells were rinsed in PBS and lysed with supplied lysis buffer for 5 min with orbital shaking. Supplied substrate solution was then added and cells were shaken for 5 min. Plates were then covered for 10 min prior to measurement of luminescence in a plate reader. An ATP standard curve was prepared in parallel using the supplied ATP solution. Results were expressed as % ATP content of vehicle-treated wells. Three to four replicates per condition per time point were performed.

2.7. Statistical analysis

Results are reported as mean ± 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 using StatView 5.0 software (SAS Institute, Cary, NC) for the Macintosh platform. Statistical significance was set at $p \leq 0.05$. Area under the curve calculations performed with Prism 4.0 software (GraphPad Software Inc., La Jolla, CA) for the Macintosh platform.

3. Results

3.1. The AMPK pathway, but not the insulin receptor pathway, is activated in C2C12 muscle cells following an 18 h treatment with extracts

In our earlier screening study (Spoor et al., 2006), it was observed that deoxyglucose uptake in the absence of insulin stimulation (basal glucose uptake) was increased in C2C12 muscle cells by extracts of three of the eight species studied here following a 1 h treatment, and by seven of the eight species following an 18 h treatment. Metformin, used as a positive control at 400 μM, had little effect after 1 h but increased uptake by approximately 30% after 18 h. At rest, glucose uptake in skeletal muscle is mediated by

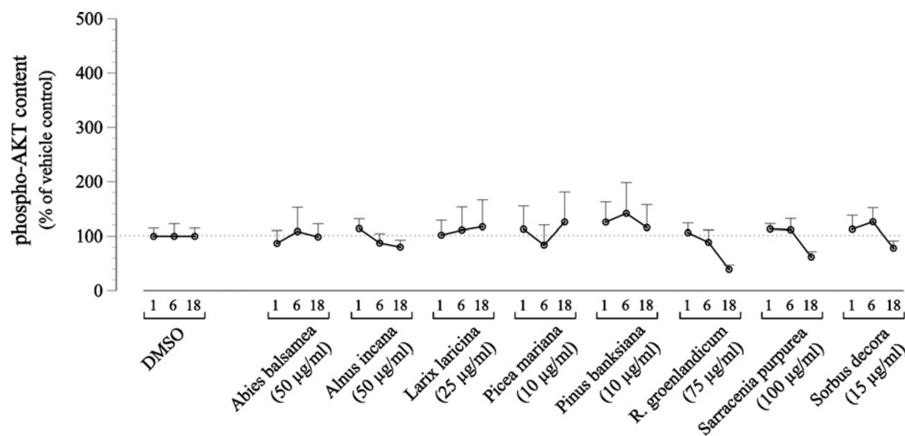

Fig. 1. Western blot analysis of phospho-Akt content in differentiated C2C12 skeletal muscle cells treated for 1, 6, or 18 h with respective plant products at the indicated dose or with vehicle control (0.1% DMSO) demonstrates that this marker of the insulin receptor signaling pathway is not phosphorylated in response to the treatments. Revelation and quantification of films were performed as described in Section 2. For each treatment duration, results are expressed relative to the respective vehicle control group and are presented as the mean ± SEM of three separate experiments.

GLUT1 glucose transporters constitutively expressed in the plasma membrane (Klip and Marette, 1992). In response to stimulation of the insulin receptor signaling pathway or of the AMPK pathway, uptake can be transiently increased by the translocation of GLUT4 and GLUT1 transporters to the plasma membrane (Abbud et al., 2000; Fujii et al., 2004; Krook et al., 2004), and possibly by the release of inhibition of the intrinsic activity of these transporters (Michelle Furtado et al., 2003). Activation of AMPK can also upregulate transport capacity (McGee and Hargreaves, 2006, 2008). To determine whether the effects of the active plant products were mediated through the insulin signaling pathway or through the AMPK pathway, C2C12 cells were treated for 1, 6, or 18 h with respective extracts and cell lysates were analyzed by western immunoblot for increased content of serine 473-phosphorylated Akt (protein kinase B), a well-recognized marker of stimulation of the insulin signaling pathway, and for increased content of threonine 172-phosphorylated AMPKalpha. The content of phospho-Akt (Fig. 1) was not increased by more than 25% above the respective vehicle control levels by any extract following either a 1 or 18 h treatment, and content was even decreased relative to control by some conditions at the later time point. As a reference, stimulation with 1 nM of insulin for 15 min results in an increase in phospho-Akt content of approximately 150%, and 45 min later this content remains elevated by approximately 75% (Benhaddou-Andaloussi et al., 2009).

In contrast to the small fluctuations in content of phospho-Akt, phospho-AMPK content (Fig. 2) underwent more important increases. Threonine 172-phosphorylated AMPKalpha content was increased by all eight extracts at the end of the 1 h treatment by 60–170% above vehicle control levels. At the end of the 6 h treatment, content remained elevated by 50% or more by six extracts, and in four cases, content was actually higher at 6 h than at 1 h. Treatment with the *Sarracenia purpurea* extract was notable in that it resulted in a 2.5-fold increase in phospho-AMPK content at this time point, an effect of similar magnitude as that of the AICAR positive control applied for 30 min. At the end of the 18 h treatment, phospho-AMPK contents were in all conditions lower than at the 6 h time point. However, in cells treated with *Sarracenia purpurea*, *Sorbus decora*, or *Alnus incana* extracts, phospho-AMPK remained 2-fold or more above the level of the vehicle control at the 18 h mark.

The same samples were also analyzed for serine 79-phosphorylated ACC content (Fig. 3), a marker of activation of the AMPK pathway. The same six extracts observed to increase phospho-AMPK content at all measured time points produced a similar pattern of increase in phospho-ACC content. In keeping

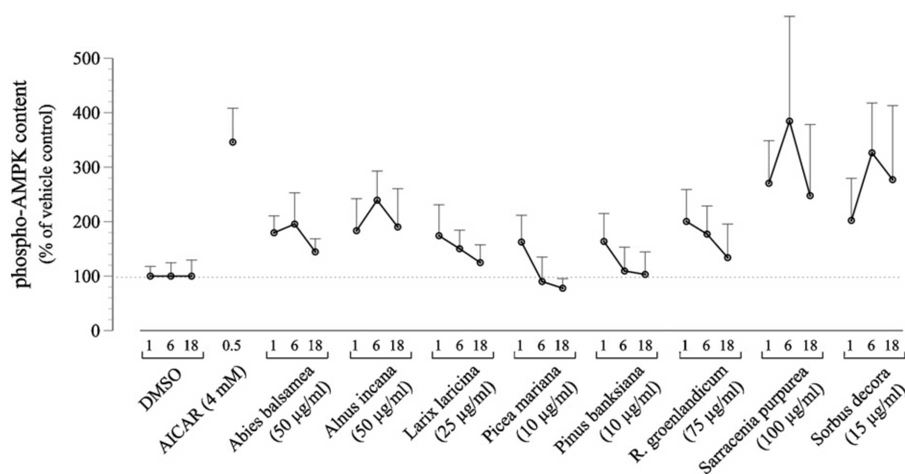

Fig. 2. Western blot analysis of phospho-AMPK content in C2C12 cells treated for 1, 6, or 18 h with respective plant products at the indicated dose or with vehicle control (0.1% DMSO) demonstrates that AMPK is phosphorylated, at least transiently, in response to treatment with all tested plant products. Quantified results are expressed relative to vehicle control and are presented as the mean ± SEM of three separate experiments for each treatment duration. The reference AMPK activator AICAR was applied for 30 min at 4 mM to a subgroup of cells treated with vehicle for 1 h.

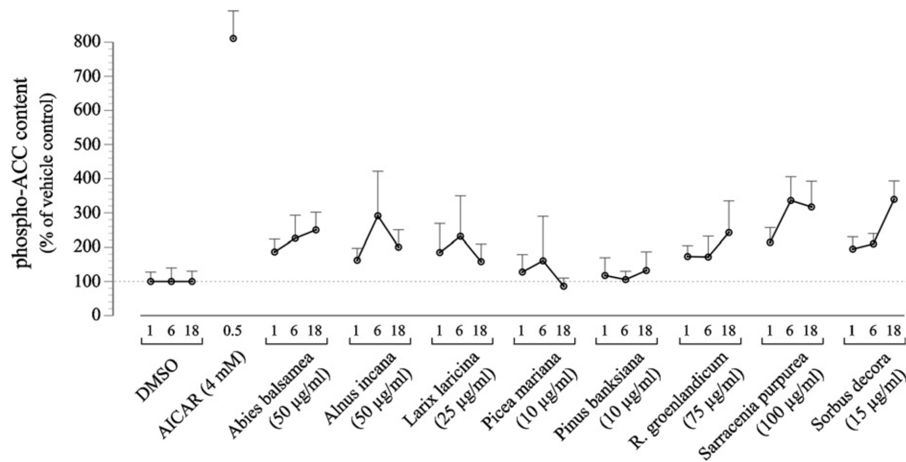

Fig. 3. Western blot analysis of phospho-ACC content in C2C12 cells treated for 1, 6, or 18 h with respective plant products at the indicated dose or with vehicle control (0.1% DMSO) demonstrates that this downstream marker of the AMPK pathway is phosphorylated in response to treatment with at least six of the tested plant products. Quantified results are expressed relative to vehicle control and are presented as the mean ± SEM of three separate experiments for each treatment duration. The reference AMPK activator AICAR was applied for 30 min at 4 mM to a subgroup of cells treated with vehicle for 1 h.

with its downstream position relative to AMPK, ACC phosphorylation was observed to be generally more sustained than AMPK activation; in cells treated with *Sarracenia purpurea*, *Sorbus decora*, *Alnus incana*, or *Rhododendron groenlandicum* extracts, contents measured at 18 h were similar to or even greater than those measured at 6 h.

3.2. A relationship between increased phospho-AMPK content and glucose uptake

Seven of the eight species studied here have previously been shown to increase glucose uptake in muscle cells in the absence of insulin stimulation following an 18 h treatment (Spoor et al., 2006). The area under the curve of phospho-AMPK content over 18 h, as defined by contents measured here at 1, 6, and 18 h (above), was observed to be well correlated (R^2 = 0.86) to the enhancement of basal glucose uptake previously reported (Fig. 4).

3.3. Extracts instantaneously disrupt function of isolated mitochondria

AMPK is highly sensitive to metabolic stress and perturbations of energy homeostasis. As many plant defensive metabolites exert disruptive effects on well-conserved energy transduction pathways (Polya, 2003), we next addressed the hypothesis that the

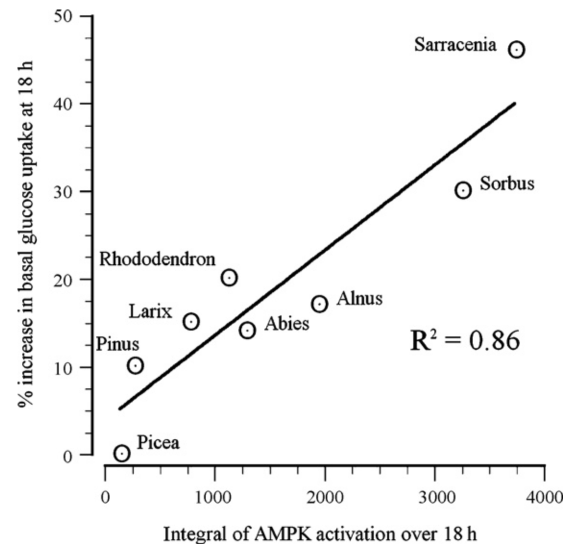

Fig. 4. AMPK phosphorylation over 18 h in C2C12 cells treated with respective plant products is well correlated with the enhancement of glucose uptake induced by an 18 h treatment with these same products. For each plant product tested, the content of phospho-AMPK measured at 1, 6, and 18 h (Fig. 2) was used to calculate an area under the curve over 18 h. Enhancement of glucose uptake in C2C12 cells was reported in our previous screening study (Spoor et al., 2006).

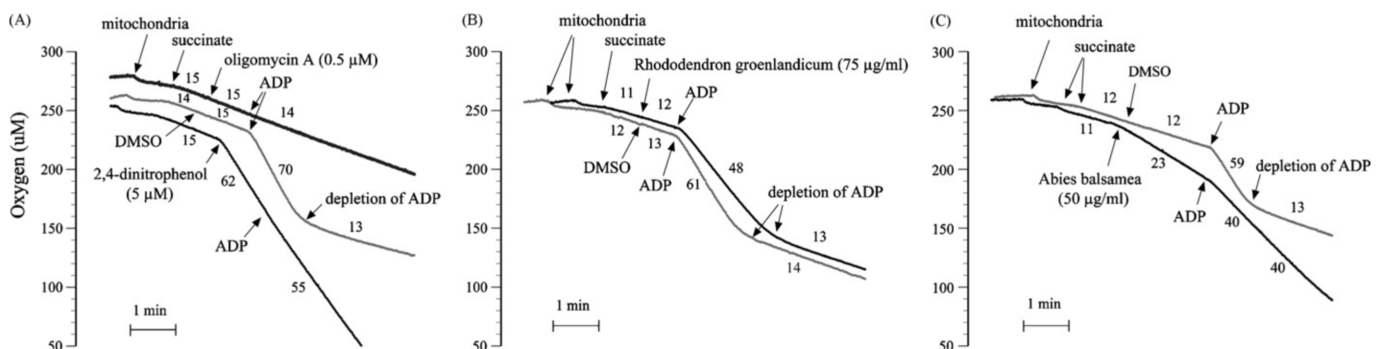

Fig. 5. Plant products induced instantaneous uncoupling- and/or inhibitory-type disruptions of oxidative phosphorylation in isolated rat liver mitochondria using succinate as substrate. Rates of O_2 consumption under basal (state 4) and ADP-stimulated (state 3) conditions were measured with a Clark-type oxygen electrode. (A) Representative tracing of the ATP synthase inhibitory effect of oligomycin A (top tracing) and of the uncoupling effect of 2,4-dinitrophenol (bottom tracing); (B) representative tracing demonstrating a purely inhibitory effect in mitochondria treated with the extract of *Rhododendron groenlandicum*. Results are summarized in Table 1; (C) representative tracing demonstrating a combination of stimulated basal O_2 consumption (consistent with an uncoupling effect) and inhibited ADP-stimulated O_2 consumption (consistent with inhibition of ATPsynthase) in mitochondria treated with extract of *Abies balsamea* as compared to vehicle-treated mitochondria.

Table 1
Disruptive effects of extracts on respiration of isolated rat liver mitochondria.

Product	Residual ATP synthetic capacity	Change in capacity attributable to uncoupling effect	Change in capacity attributable to inhibitory effect
Abies balsamea (50 μg/ml)	33 ± 5%	−23 ± 3%	−46 ± 9%
Alnus incana (50 μg/ml)	87 ± 4%	−2 ± 1%	−13 ± 4%
Larix laricina (50 μg/ml)	62 ± 8%	−16 ± 4%	−22 ± 11%
Picea mariana (50 μg/ml)	98 ± 3%	0 ± 1%	−2 ± 4%
Pinus banksiana (50 μg/ml)	89 ± 1%	−3 ± 2%	−7 ± 3%
Rhododendron groenlandicum (50 μg/ml)	80 ± 3%	0 ± 1%	−23 ± 3%
Sarracenia purpurea (50 μg/ml)	62 ± 5%	−7 ± 1%	−31 ± 4%
Sorbus decora (50 μg/ml)	82 ± 2%	−3 ± 1%	−17 ± 2%
2,4-Dinitrophenol (5 μM)	0 ± 0%	−100 ± 0%	0 ± 0%
Oligomycin A (0.5 μM)	0 ± 0%	0 ± 0%	−100 ± 0%

Values are mean ± SEM for three to four separate experiments with two to three replicates each.

extracts found to activate the AMPK pathway would also disrupt mitochondrial function. Succinate-supported rates of basal and ADP-stimulated oxygen consumption were measured in isolated rat liver mitochondria treated with either vehicle or with respective plant extracts at the same concentration used to induce glucose uptake and stimulation of AMPK. An increase in RBOC reflects an uncoupler-type disruption of mitochondrial function, as can be induced with the reference compound 2,4-dinitrophenol (Fig. 5A). A decrease in RASOC, occurring in the absence of any change in RBOC, typically reflects an inhibition of ATP synthase, as can be induced with the reference compound oligomycin A (Fig. 5A). Concurrent uncoupling and inhibition of ATP synthase can occur, especially when testing complex mixtures possibly containing multiple active principles. However, concurrent effects on RBOC and RASOC can also indicate other types of disruption, such as an inhibition of substrate transport across the inner mitochondrial membrane. Of the eight plant products tested here, six induced a concurrent increase in RBOC and decrease in RASOC, whereas one, the *Rhododendron groenlandicum* extract, affected RASOC only, and one, the *Picea mariana* extract, had no effect on mitochondrial function. The seven products found to affect mitochondrial function were therefore the same seven previously observed to enhance glucose uptake (Spoor et al., 2006). These disruptions, whether concurrent and therefore cumulative, or of a single type, resulted in reductions in mitochondrial capacity for ATP synthesis ranging from 11% to 67% (Table 1). All disruptions of mitochondrial function observed were instantaneous. Representative oxygen consumption tracings are shown for the extract of *Rhododendron groenlandicum* (Fig. 5B) affecting only RASOC, and for the extract of *Abies balsamea* (Fig. 5C), the product that induced the greatest disruptions (23% and 46% reductions in mitochondrial capacity by uncoupling and inhibitory effects, respectively).

3.4. Extracts stimulate the rate of acidification of the cell culture medium

When mitochondrial capacity for ATP synthesis is reduced to the point where it can no longer meet the energy needs of the cell, dependence on anaerobic glycolysis for energy production is increased. By measuring flux through glycolysis, it is therefore possible to obtain information concerning the degree of metabolic stress induced by the plant products as well as information about their rapidity and duration of action. Furthermore, these experiments allow for the comparison of effects across different cell types. As lactic acid is the end product of anaerobic glycolysis, flux through this pathway was measured as the rate of acidification of cell culture medium. Extracts were applied to cells for 4 h, over which time the medium was periodically sampled and pH determined spectrophotometrically using Phenol Red as indicator. Results were expressed in the form of cumulative secretion of acid equivalents over time (Fig. 6). All extracts were observed to increase the rate of acidification by 2–3-fold over the first 20 min of the assay in C2C12 cells. However, beyond this time, effects varied greatly between the different extracts. A sustained increase in the rate of acidification over the 4 h experimental period was produced only by the *Larix laricina* and *Abies balsamea* extracts, the two extracts that most severely disrupted function in isolated mitochondria. The *Alnus incana* extract produced the smallest effect, which lasted no longer than 20 min. The *Picea mariana* extract, despite having no observable effect in isolated liver mitochondria, produced the largest increase in rate of acidification; however, this increase was not sustained beyond the first hour. The *Sorbus decora*, the *Rhododendron groenlandicum*, and the *Sarracenia purpurea* extracts produced short-lived effects. Interestingly, the latter two demonstrated a tendency to depress anaerobic metabolism below the vehicle control rate of acidification after the first hour of treatment. In H4IIE hepatocytes, slightly different effects were observed. Notably, the *Picea mariana* extract had no effect in this cell line. Also, the magnitude of effect of the *Abies balsamea* was much greater in hepatocytes than muscle cells, surpassing the effect of the positive control FCCP. Finally, in some instances, rate of acidification was observed to be paradoxically depressed rather than stimulated.

3.5. Extracts induce a depression of cytosolic ATP content

Similarly to the acidification assay above, the concentration of cytosolic ATP was assessed in order to gain insight into magnitude, rapidity of onset, and duration of the metabolic stress induced by the plant extracts in whole cells. In C2C12 cells, all species, with the exception of *Rhododendron groenlandicum*, were observed to induce a depression of cytosolic ATP content 1 h into the treatment (Fig. 7). In the case of the *Larix laricina* and *Abies balsamea* extracts, this depression was sustained over the 6 h experimental period, concordant with their sustained increase in anaerobic metabolism observed in the acidification assay. The effect of the *Sorbus decora* extract was the shortest lived. The effects of other extracts were normalized by the 6 h time point. The *Rhododendron groenlandicum* and *Sorbus decora* extracts tended to induce supranormal ATP levels, possibly due to the overcompensation of ATP synthesis in response to metabolic stress and activation of AMPK. Supranormal ATP content by these extracts coincided with a tendency for inhibition of Akt activity (Fig. 1).

As in the acidification assay, cell-type differences were observed. Notably, the magnitude of effect of the extracts was smaller in H4IIE hepatocytes; only the *Larix laricina* extract induced a depression between 1 and 6 h (Fig. 7). However, the overcompensation phenomenon described above was observed to be induced by five extracts as well as by the FCCP positive control. The two most important overcompensations, those induced by the *Sarracenia purpurea* and *Sorbus decora* extracts, concord with the observed depression of anaerobic metabolism induced by these products

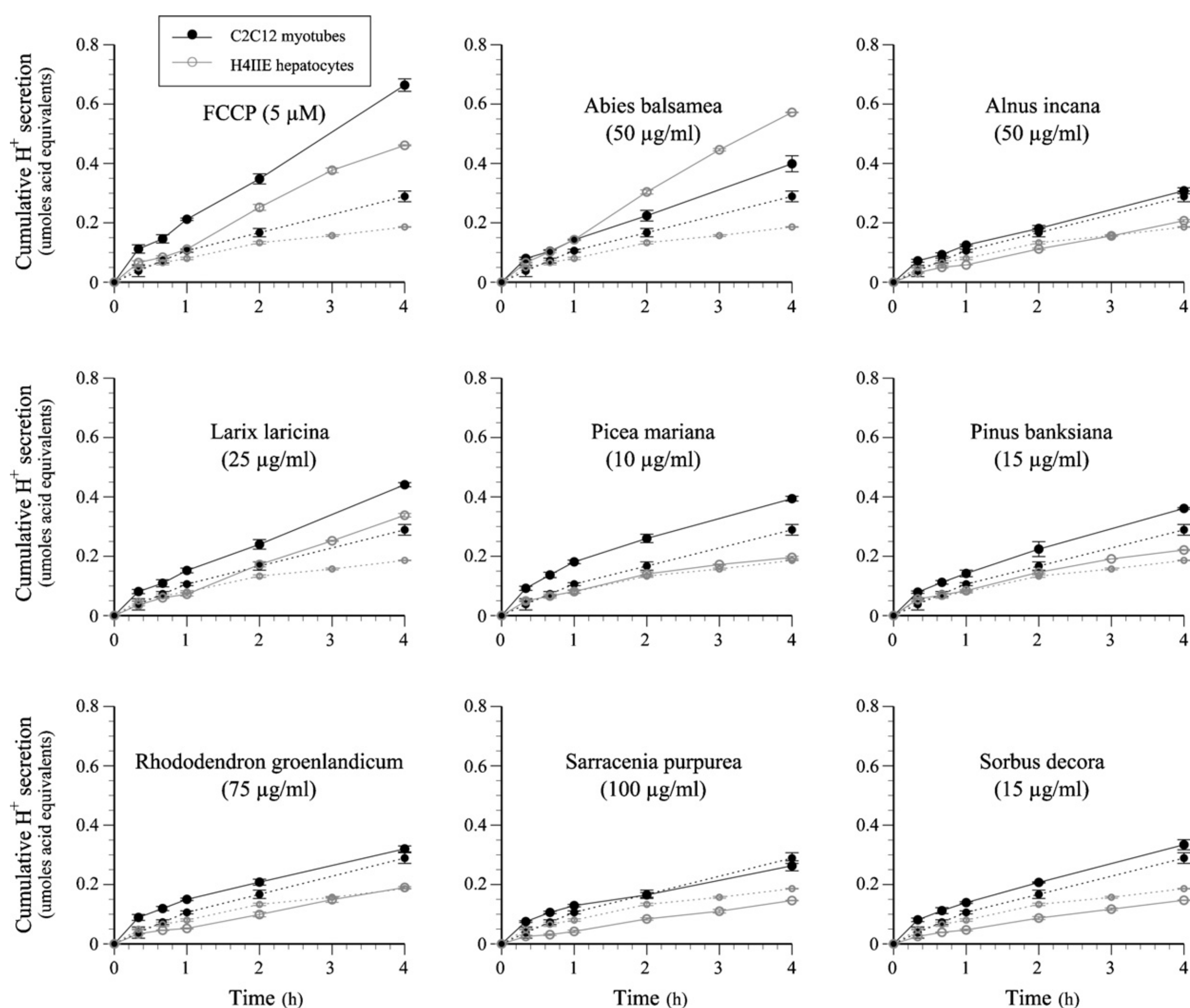

Fig. 6. The secretion of acid equivalents into the culture medium, a reflection of flux through anaerobic glycolysis, was affected by treatment with respective plant products in C2C12 skeletal muscle cells (closed circles) and in H4IIE liver cells (open circles). The artificial uncoupler FCCP was used as a positive control. The vehicle control group is represented by dashed lines in each plot. Results were expressed as mean ± SEM cumulative secretion of acid equivalents (μmol). Four to five replicates were performed per condition per time point.

(Fig. 6), which may indicate allosteric inhibition of glycolysis by surplus ATP.

4. Discussion

Medicinal plant products with anti-hyperglycemic activity are common in the traditional medicine of cultures throughout the world, and the efficacy of many has been rigorously studied (Haddad et al., 2006; Marles and Farnsworth, 1995). The cellular and molecular mechanisms of action of these, however, have generally not been elucidated. The present study focuses on plant products selected from the pharmacopeia of an aboriginal Canadian population, the Cree of Iiyiyiu Istchii, and was undertaken for further evaluation of these products as promising and culturally adapted treatment options for diabetes care in susceptible aboriginal populations. Specifically, the present study addressed the mechanism of action through which these products enhance the rate of basal glucose uptake in a model skeletal muscle cell line, an activity first observed in our recent screening study of the antidiabetic potential of these products (Spoor et al., 2006).

Our results demonstrate that the selected medicinal plant products act through a common mechanism involving the AMPK signaling pathway, rather than through the insulin receptor pathway, to increase basal glucose uptake in C2C12 skeletal muscle cells following 18 h of treatment. Indeed, whereas no significant increases in the content of phosphorylated Akt were observed throughout the 18 h period, phosphorylation of AMPK and ACC were increased up to 2.5- and 3.5-fold, respectively. The AMPK pathway converges with the insulin receptor pathway at the level of AS160 and in this way can induce the translocation of GLUT4 glucose transporters to the sarcolemma and promote an increase in the rate of uptake (Cartee and Wojtaszewski, 2007; Thong et al., 2007). Direct effects of the AMPK pathway on the intrinsic activity of constitutively expressed GLUT1 transporters and of GLUT4 transporters have also been reported (Michelle Furtado et al., 2003). However, increase in the expression of glucose transporters and of other effectors of the insulin receptor pathway that are mediated by AMPK signaling (McGee and Hargreaves, 2008) likely play a more important role in the long-lasting effects of the products studied herein. This was supported by the observation that some plant products enhanced basal glucose uptake to levels surpassing the maximal acute stimulatory effect of insulin (Spoor et al., 2006). This is now also supported by an excellent correlation between the enhancement of glucose uptake after the 18 h treatment and the activation of AMPK over the entire treatment period (Fig. 5). Arguments against residual acute stimulation of uptake at 18 h, such as the observation that the effect of insulin applied acutely was addi-

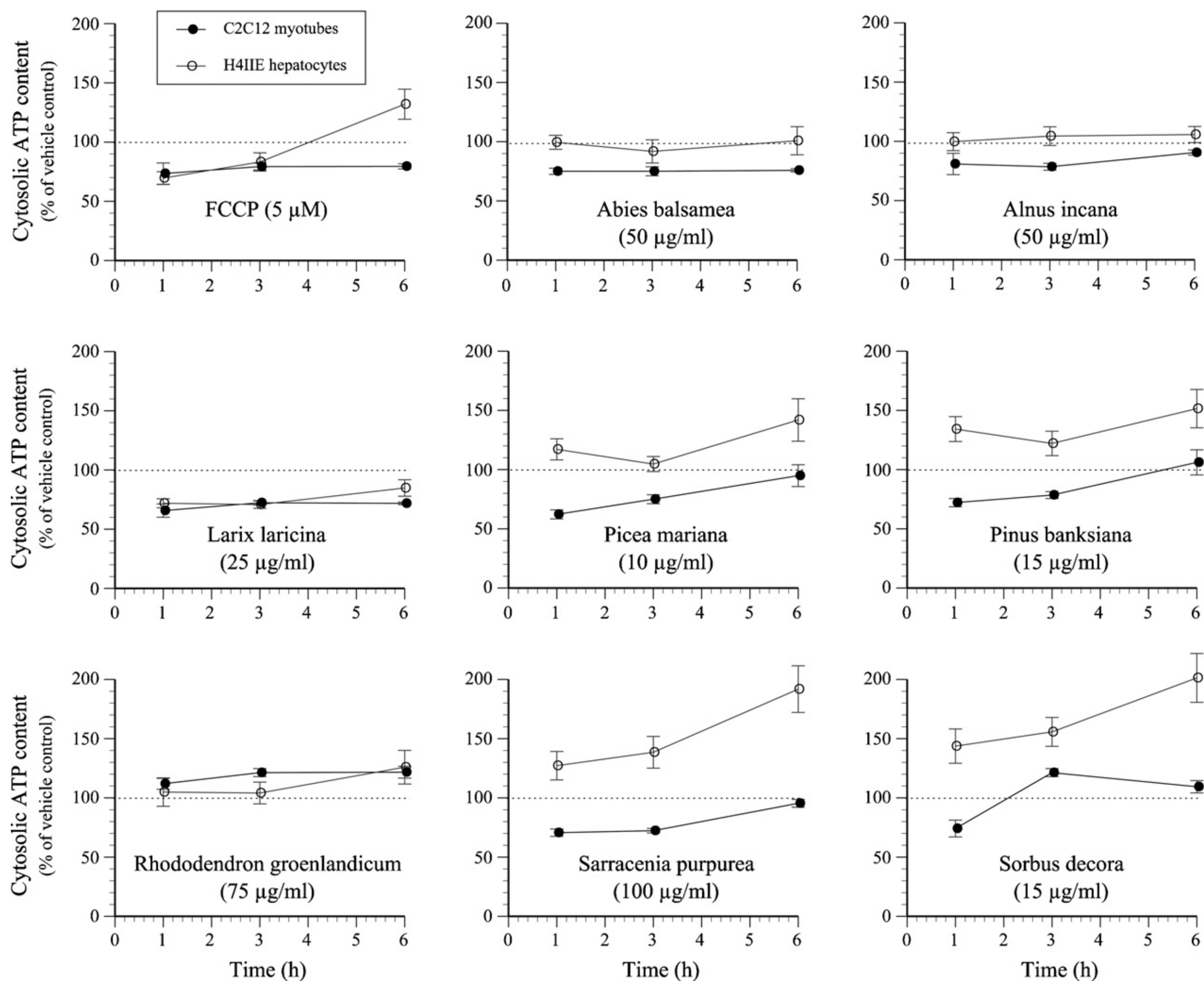

Fig. 7. The cytosolic concentration of ATP was affected by treatment with respective plant products in C2C12 skeletal muscle cells (closed circles) and in H4IIE liver cells (open circles). The artificial uncoupler FCCP was used as a positive control. Results are expressed relative to the vehicle control group and represent mean ± SEM of three to four replicates per condition per time point.

tive to the effect induced by 18 h treatment with plant products (Spoor et al., 2006), now also include the finding that AMPK activation was either declining or had returned to control levels at the 18 h time point.

This activation of the AMPK pathway can be explained by the induction of a metabolic stress through a mild and transient disruption of mitochondrial energy transduction (Hayashi et al., 2000). Indeed, all seven plant products previously shown to stimulate glucose uptake were here found to induce in isolated mitochondria an inhibitory-type disruption of oxidative phosphorylation or a combination of inhibition and uncoupling-type disruptions. Metabolic stress was confirmed in intact cells as increased flux through anaerobic glycolysis and depression of cytosolic ATP concentrations. Both inhibitory and uncoupling effects additively contribute to a reduction of mitochondrial capacity. When capacity is no longer sufficient to meet energy demand, energy homeostasis is disturbed and activation of AMPK then results from the simultaneous drop in ATP concentration and rise in AMP concentration (Winder and Thomson, 2007). In the face of such metabolic stress, the function of AMPK is to restore energy homeostasis as well as to protect the cell against future metabolic stress. This is accomplished by acutely increasing glucose and fat uptake to support a coordinated stimulation of carbohydrate and lipid oxidation, while at the same time transiently inhibiting energy-consuming synthetic processes (Winder and Thomson, 2007). The adaptive effects of AMPK include an increase in mitochondrial capacity through mitochondrial biogenesis and an increase in substrate uptake capacity (McGee and Hargreaves, 2008; Reznick and Shulman, 2006; Winder, 2001).

Induction of metabolic stress through disruptions of mitochondrial function has long been known to produce cytoprotective effects. For example, the classical uncoupler 2,4-dinitrophenol has been shown to increase glucose uptake in muscle cells (Bashan et al., 1993), and sodium azide, a cytochrome oxidase inhibitor, has been shown to rapidly upregulate GLUT1 expression by several folds in liver cells (Behrooz and Ismail-Beigi, 1997; Shetty et al., 1993). The effects of the commonly prescribed hypoglycemic drug Metformin are believed to be mediated similarly through inhibition of complex I of the electron transport chain (El-Mir et al., 2000; Owen et al., 2000) and subsequent activation of AMPK (Hayashi et al., 2000).

It is remarkable that products from such a wide variety of plant species, as are represented here, share a common mechanism of action. However, disruption of energy transduction and the resulting indirect activation of AMPK represent a simple mechanism requiring less molecular specificity than the activation of the insulin receptor signaling pathway or even than the inhibition of negative regulators of this pathway. Therefore, it is possible that the metabolic stress mechanism is widespread and may explain the antidiabetic activity of traditional medicines from various cultures. This mechanism is also in accordance with the defensive role of many plant secondary metabolites, evolved for fungicidal, bactericidal, and insecticidal activities, all mediated by the targeting

of highly conserved energy transduction pathways (Polya, 2003). Indeed, several plant compounds, including many flavonoids, are known disruptors of mitochondrial oxidative phosphorylation, acting either as uncouplers or as inhibitors (Polya, 2003).

Targeting mitochondrial energy transduction is inherently dangerous; increased reliance on anaerobic metabolism as a compensation for compromised mitochondrial capacity results in an increase in the rate of release of hydrogen ions, which can lead to systemic acidosis. Metabolic acidosis is indeed the primary danger associated with the use of biguanides and represents a potential complication that needs to be considered in the case of the plant products tested herein. This danger can clearly be minimized if the metabolic stress is short-lived. The results obtained in our study on the rate of acidification and on cytosolic ATP content suggest that the effects of the plant products are indeed in most cases short-lived and of low magnitude, aerobic capacity generally being restored after 30–60 min. Such effects would be expected with rapidly metabolized products and future studies, notably on active principles, will need to address this point.

Our results highlight the importance of duration of AMPK activation for maximizing the enhancement of glucose uptake; the products that were notable in their sustained activation of this pathway, the extracts of *Sarracenia purpurea* and *Sorbus decora*, were also the most efficacious for increasing uptake. A less intuitive observation, however, is that there appears to be an optimal pattern of metabolic stress for enhancing muscle glucose uptake (and perhaps for maximizing other insulin-like effects of AMPK such as the inhibition of hepatic glucose output) and that sustained metabolic stress may actually be counterproductive. Indeed, the products that induced the most prolonged stress, as gauged by the depression of ATP concentration and increased flux through anaerobic glycolysis, were not the most efficacious for activating AMPK or enhancing uptake. On the other hand, the extracts of *Sarracenia purpurea* and of *Sorbus decora* produced a paradoxical combination of sustained activation of the AMPK pathway and short-lived metabolic stress. This observation therefore suggests the possibility that the potential for lactic acidosis can be uncoupled from AMPK-stimulating activity. This augers well for efforts aimed at identifying better alternatives to Metformin and the biguanides.

We also observed that depression of ATP concentration was in several cases followed by a compensatory overshoot phenomenon. This provides further evidence that, for the most part, the extracts induce a sudden but short-lived metabolic stress. As an overcompensation of ATP synthesis can be explained by an AMPK-triggered stimulation of lipid and carbohydrate oxidation that is sustained several hours after the restoration of homeostasis, the concordance of these overshoots with sustained ACC phosphorylation is further support of a mismatch between the kinetics of metabolic stress and those of AMPK activation. It should be noted that ATP overcompensation and/or sustained ACC phosphorylation induced by the *Rhododendron groenlandicum*, *Sorbus decora*, and *Sarracenia purpurea* extracts also coincided with depressed glycolytic flux and inhibition of the insulin signaling pathway, expected effects in the face of energy surfeit.

Finally, it is noteworthy that cell-type specific effects were observed in the acidification and cytosolic ATP assays. A striking example was that of the *Picea mariana* extract which had no effect on acidification in liver cells, concordant with its absence of effect on isolated liver mitochondria, and yet produced the highest measured rate of acidification in muscle cells. Such cell-type specificities, possibly due to molecular differences in the electron transport chain, support the hypothesis that it is possible to target AMPK-mediated effects only in some tissues while sparing others. This is highly desirable from a therapeutic point of view since in pancreatic beta cells, the activation of AMPK can decrease insulin secretion (da Silva Xavier et al., 2003), while in hypothalamic cells, it can trigger hunger signaling (Kola, 2008). In our previous screening study (Spoor et al., 2006), it was observed that the *Larix laricina* and *Sorbus decora* extracts had no effect on glucose uptake in 3T3-L1 adipocytes, and only the *Abies balsamea*, *Larix laricina*, *Rhododendron groenlandicum*, and *Sarracenia purpurea* extracts inhibited glucose-stimulated insulin secretion in β cells.

In conclusion, the medicinal plant species studied herein exert their activity through a common mechanism similar to that of Metformin, involving the activation of AMPK secondary to metabolic stress induced by the disruption of mitochondrial energy transduction. Due to its simplicity, this mechanism is likely to apply to other antidiabetic plant products used elsewhere in the world. The active products studied herein may be useful against insulin resistance and metabolic diseases. Testing for antihyperglycemic activity in animal models is currently underway, as is the isolation and identification of the active constituents of these products. The detailed profile of biological activity obtained in this study will help inform the choice of candidate products for integration into the diabetes care of Canadian aboriginal populations.

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ORIGINAL ARTICLE

Multiple molecular targets underlie the antidiabetic effect of *Nigella sativa* seed extract in skeletal muscle, adipocyte and liver cells

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Aim: *Nigella sativa* (*N. sativa*) is a plant widely used in traditional medicine of North African countries. During the last decade, several studies have shown that extracts from the seeds of *N. sativa* have antidiabetic effects.
Methods: Our group has recently demonstrated that *N. sativa* seed ethanol extract (NSE) induces an important insulin-like stimulation of glucose uptake in C2C12 skeletal muscle cells and 3T3-L1 adipocytes following an 18 h treatment. The purpose of the present study was to elucidate the pathways mediating this insulin-like effect and the mechanisms through which these pathways are activated.
Results: Results from western immunoblot experiments indicate that in C2C12 cells as well as in H4IIE hepatocytes, but not in 3T3-L1 cells, NSE increases activity of Akt, a key mediator of the effects of insulin, and activity of AMP-activated protein kinase (AMPK), a master metabolic regulating enzyme. To test whether the activation of AMPK resulted from a disruption of mitochondrial function, the effects of NSE on oxygen consumption were assessed in isolated liver mitochondria. NSE was found to exhibit potent uncoupling activity.
Conclusion: Finally, to provide an explanation for the effects of NSE in adipocytes, PPARγ stimulating activity was tested using a reporter gene assay. Results indicate that NSE behaves as an agonist of PPARγ. The data supports the ethnobotanical use of *N. sativa* seed oil as a treatment for diabetes, and suggests potential uses of this product, or compounds derived thereof, against obesity and the metabolic syndrome.

Keywords: 3T3-L1 adipocytes, AMP-activated protein kinase, Akt/protein kinase B, C2C12 skeletal muscle cells, H4IIE hepatocytes, intracellular signalling, mitochondrial respiration, peroxisome proliferator-activated receptor γ, type 2 diabetes mellitus, uncoupling

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Introduction

Type 2 diabetes (T2D) is a serious health problem in most developed and developing countries [1]. The World Health Organization estimates that 180 million people worldwide are afflicted and that this number will double by 2030 [1]. Pharmaceutical interventions for T2D are essentially limited to the sulfonureas, the biguanides and the thiazolidinediones. The sulfonureas address the problem indirectly by stimulating secretion of insulin [2]. The biguanides target liver, and to a lesser extent skeletal muscle, where they stimulate the AMP-activated protein kinase (AMPK) pathway [3], an insulin-independent signalling cascade inducing the inhibition of hepatic glucose output and the stimulation of muscle glucose uptake [4]. Finally, the thiazolidinediones exert their effect primarily on the adipose tissue, where they activate the peroxisome proliferator-activated receptor (PPAR)γ nuclear receptors [5] and upregulate the expression of effectors of insulin.

Medicinal plants with antihyperglycaemic activity are found in the traditional medicine of numerous cultures throughout the world [6, 7]. Today, such remedies remain important in many cultures despite the general lack of scientific study. However, when plant products have been studied in preclinical and clinical settings, most have been shown to be efficacious [8]. In several cases, the AMPK pathway is emerging as a potential common mediator of their activity [9–11].

Nigella sativa L. (*N. sativa*) is one such medicinal plant that has stood the test of time; the seeds of this herb having been used extensively in Middle Eastern and Northern African countries for over 2 millennia to treat diabetes and several other ailments [12–14]. Its antihyperglycaemic activity has been validated by several studies [15–30]. Our group has recently demonstrated that *N. sativa* seed extract (NSE) exhibits the remarkable combined ability to increase insulin secretion, induce proliferation of pancreatic β-cells and stimulate glucose

*These authors contributed equally to this work.

uptake in skeletal muscle cells and adipocytes [30]. The goal of the present study was to elucidate the molecular targets mediating the extrapancreatic effects of NSE. Our results demonstrate that this plant product possesses the unique property of activating the AMPK pathway, the insulin signalling pathway as well as PPARγ.

Material and Methods

N. sativa Plant Material and Constituent Compounds

Seeds of *N. sativa* were obtained from an herbalist in Rabat, Morocco in August 2005 and properly authenticated (Prof. A. Oulyahya, botanist, Institut Scientifique, Rabat, Morocco; voucher 10359). Seeds were washed, dried and then powdered with an electric micronizer. Powder was extracted three times with 80% ethanol and solvent evaporated at 40 °C under reduced pressure, resulting in a crude extract characterized by an oily phase and a solid phase. Fractions were also prepared from *N. sativa* seed powder by sequential extraction with solvents of increasing polarity, namely hexane, ethyl acetate, methanol, and 70% methanol/30% water, and with evaporation as above. The crude extract and the fractions were conserved at 4 °C and protected from light and humidity; all were solubilized freshly in dimethyl sulfoxide (DMSO) prior to treatment of cells (below). The oily and solid phases of the crude extract were solubilized at a proportion of 70 and 30% (w/v), respectively, in accordance with their yield; this product is henceforth referred to as *Nigella* seed extract (NSE).

Some constituent compounds of *N. sativa* seeds were also tested for biological activity. These included thymol (CAS No. 89-83-8) [31], carvacrol (CAS No. 499-75-2) [32] and thymoquinone (CAS No. 490-91-5) [18], purchased from Sigma-Aldrich (St. Louis, MO, USA); hederagenin (CAS No. 465-99-6) [33], purchased from Enquce Biochemicals (Kamsack, SK, Canada) and nigellimine (CAS No. 4594-02-9) [34], a gift from Dr Tony Durst (University of Ottawa, Ottawa, ON, Canada).

Reagents, Cell Lines and Antibodies

Reagents were purchased from Sigma-Aldrich, unless otherwise noted. Cell culture medium was from Wisent (Saint-Bruno, QC, Canada). Fetal bovine serum (FBS), antibiotics and trypsin were from Invitrogen Life Technologies (Carlsbad, CA, USA). Culture plates were from Corning (New York, NY, USA). C2C12 murine myoblasts, H4IIE hepatocytes and 3T3-L1 murine preadipocytes were obtained from ATCC (Manassas, VA, USA). Antibodies directed against phosphorylated Akt (Ser 473), extracellular signal-regulated kinases (ERK)1/2 (Thr 202/Tyr 204), AMPKα (Thr 172) and acetyl CoA carboxylase (ACC) (Ser 79) as well as corresponding pan-specific antibodies were from Cell Signaling Technology (Danvers, MA, USA). Secondary horseradish peroxidase (HRP)-conjugated antibodies were from Jackson Immunoresearch (West Grove, PA, USA). Protein assay reagents were from Pierce Protein Research Products (Thermo Scientific, Rockford, IL, USA).

Cell Culture

C2C12 myoblasts were cultured as previously described [30, 35] in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine seum (FBS), 10% horse serum (HS) and antibiotics (penicillin 100 UI/ml, streptomycin 100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Upon reaching 80% confluence, serum content was reduced to 2% HS and experiments performed on well-differentiated multinucleated cells 6–7 days later. 3T3-L1 preadipocytes were grown as previously described [30, 35] in DMEM supplemented with 10% FBS and antibiotics until 80% confluent. Differentiation into adipocytes was induced by adding 500 μM 3-isobutyl-1-methylxanthine, 10 μM dexamethasone and 500 nM insulin to the medium for 2 days. Experiments were performed when more than 90% of cells exhibited lipid droplets, typically after 10–14 days in an insulin-supplemented medium. H4IIE hepatocytes were grown in DMEM containing 10% HS until confluent and experiments performed 1–3 days later [36]. All cells were treated with 200 μg/ml of NSE, the maximal soluble concentration in DMSO. This concentration was non-toxic in all cell lines used for up to 24 h.

Western Immunoblot

C2C12 myotubes, 3T3-L1 adipocytes and H4IIE hepatocytes were cultured in 6-well plates and treated with NSE for 18 h in conditions similar to those used previously to assess the effects of NSE on glucose uptake [30], with the following modifications. For immunoblot analysis of phospho-Akt and ERK1/2, the last 3 h of treatment were performed in serum-free medium containing fresh extract. Cells treated with extract or vehicle (DMSO 0.1%) were incubated 5 min at 37 °C with 0, 1 or 100 nM insulin dissolved in 0.01 M HCl. For immunoblot analysis of phospho-AMPK and ACC, cells were treated with NSE or 0.1% DMSO in complete medium with no change of medium prior to lysis.

-Aminoimidazole-4-carboxamide-1-b-D-rubofuranozide (AICAR; Toronto Research Chemicals, North York, ON, Canada) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were used as positive controls for AMPK activation. AICAR solubilized in water was added to DMSO-treated cells for 30 min at 2 mM. FCCP was used for 1 h at 5 μM. For insulin time–course experiments, parallel plates of cells were washed free of insulin in warm serum-free growth medium and incubated for an additional 10, 25, 55 or 85 min. Following treatment, plates were placed on ice and immediately washed three times in an ice-cold phosphate-buffered saline (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, 0.1% SDS) containing a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany) supplemented with 1 mM phenylmethanesulfonyl fluoride, as well as a phosphatase inhibitor cocktail (1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride). Cells were lysed for 15 min on ice, and were then scraped into microcentrifuge tubes, vortexed and centrifuged at 12 000 *g* for 10 min at 4 °C. Supernatants were decanted and stored at

−80 °C until further analysis. The lipid layer of 3T3-L1 cells was aspirated prior to decanting of supernatant. Protein content was assayed by the bicinchoninic acid method standardized to bovine serum albumin.

Lysates were diluted to a concentration of 1.25 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). Twenty micrograms of protein of each sample was separated on 10% polyacrylamide mini-gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buferred saline (20 mM Tris–HCl, pH 7.6 and 137 mM NaCl) containing 0.1% Tween-20 (TBST), then incubated overnight at 4 °C in blocking buffer with primary antibodies at a concentration of 1 : 1000 in TBST plus 5% milk. Membranes were washed five times with TBST and incubated for 90 min at ambient temperature in TBST with appropriate HRP-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 three different passages of cells, each passage containing all conditions in parallel. Densitometric analysis of films was performed using a flatbed scanner (Hewlett Packard, ScanJet 6100C/T, Palo Alto, CA, USA) and NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Isolation of Mitochondria and Measurement of Respiration

Mitochondria were isolated from the liver of 225–250 g Sprague-Dawley rats (Charles River, St-Constant, QC, Canada). 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 Animal Care. Mitochondria were isolated from 1 g of liver following the method of Johnson and Lardy [37], as previously described in detail [38].

Oxygen concentration was measured at 25 °C at a frequency of 1 Hz using a Clark-type oxygen microelectrode in a 1 ml temperature-controlled chamber (Oxygraph System, Hansatech Instruments, Norfolk, England), as previously described in detail [38, 39]. Mitochondrial respiration was initiated by addition of the complex II substrate succinate (5 mM final concentration). After reaching a stable rate of state 4 respiration, 2 µl of NSE or vehicle solution (0.1% DMSO) was injected for final concentrations of 25–200 µg/ml of NSE. Six fractions and a number of purified constituents of *N. sativa* seed were also tested. After at least 30 additional seconds, oxidative phosphorylation and state 3 respiration were induced with ADP (250 µM final concentration). In some experiments, mitochondria were pretreated with 2 µM of atractyloside potassium salt (AK), an inhibitor of the adenine nucleotide translocase (ANT), to explore the implication of this transporter in the action of NSE. Increase in basal rate of O_2 consumption, an index of uncoupling, was calculated from each experiment by subtracting the rate measured in the presence of extract from the rate measured in the absence of extract and dividing the difference by the latter rate. Decrease in ADP-stimulated O_2 consumption rate, an index of inhibition of oxidative phosphorylation (i.e. ATP synthase), was calculated by subtracting the rate measured in the extract experiment by the average rate measured in the vehicle control experiments of the corresponding experimental session, and dividing the difference by this average control rate.

PPARγ Activation Experiments

The following experiments were performed by Indigo Biosciences (State College, PA, USA). Human embryonic kidney (HEK) 293-T fibroblasts (ATCC, Manassas, VA, USA) were cultured in high-glucose DMEM supplemented with 10% FBS and antibiotics and transiently transfected with Gal4-human PPARγ and Gal4-luciferase plasmid DNA. Plasmid integrity and transfection efficiency were carefully verified. Cells at 80% confluence in 100-mm culture dishes were transfected for 6 h using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by overnight incubation. The media was then replaced with fresh media containing NSE or positive control in DMSO (0.1% final concentration). Fourteen hours after treatment, the cells were lysed with passive lysis buffer (Promega, Madison, WI, USA) for 30 min; luciferase and renilla activity were measured with the Luciferase dual reporter assay kit (Promega) following the manufacturer's instructions and using a Tecan GeniosPro plate reader (Research Triangle Park, NC, USA). The fold induction of normalized luciferase activity was calculated relative to DMSO vehicle-treated cells, and represents the mean of three independent samples per treatment group. For agonist experiments, cells were treated with NSE (200, 50, 12.5, 3.13 and 0.78 µg/ml) or with positive control (PPARγ-agonist rosiglitazone; 20, 2, 0.2, 0.02 and 0.002 µM).

Glucose Transport Assay

Differentiated C2C12 cells grown in 12-well culture plates were treated with 200 µg/ml of NSE, thymoquinone or vehicle control (0.1% DMSO) for 18 h. The glucose transport assay has been described in detail elsewhere [30]. Four replicates were performed for each experimental condition.

Rate of Acidification of Extracellular Medium

A spectrophotometric assay of change in cell culture medium pH over time, quantitative between pH 7.2 and 6.4, was developed based on similar assays [40]. The assay medium consisted of Dulbecco's PBS containing Phenol Red as a pH indicator and modified to contain a total of 2 mM phosphate (for approximately 20% of the buffering capacity of Dulbecco's original recipe). The composition of this modified Dulbecco's PBS (mD-PBS) was 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 (Sigma-Aldrich; Phenol Red 0.5% solution) and deionized ultrafiltered water (Fisher Scientific, Ottawa, ON, Canada), which was adjusted to 7.20 at ambient temperature with NaOH immediately prior to

the assay. Absorbance of 100 µl samples transferred to 96-well plates (Sarstedt Montreal, QC, Canada) was measured at ambient temperature at 530 and 450 nm using a Wallac Victor 2 plate reader (Perkin-Elmer, Waltham, MA, USA) and the ratio of abs 530/abs 450 was calculated. The relationship between pH and the log of this ratio was observed to be linear over the range of pH of 6.4–7.4, in agreement with a pKa of 6.9 for balanced salt phosphate buffers. The following function was used to model the relationship between pH and absorbance over the pH range of 6.4–7.2 : $pH = 0.765 \ast \ln(abs\ 530/abs\ 450) + 7.61 (r^2 = 0.99)$. The buffering capacity of mD-PBS was observed to be nearly linear between pH 6.2 and 7.2 and was calculated to be 1.075 mM equivalents per pH units between 6.3 and 7.1.

Experiments were performed on 7-day differentiated C2C12 muscle cells and on 1-day postconfluent H4IIE liver cells grown in 12-well plates. On the day of the experiment, cells were 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 prewarmed 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. 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. Because DMSO was observed to stimulate acidification, as noted by others [41], extracts were solubilized in 80% ethanol at 1000× their final concentration, for a final ethanol concentration of 0.08%. All treatments were adjusted to pH 7.2 separately immediately prior to the assay. FCCP solubilized in ethanol was used at 5 µM as a positive control. Results were expressed as cumulative secretion of acid equivalents (micromoles) for two experiments of four to five replicates per condition per time-point.

Cytosolic ATP Assays

Total cytosolic ATP was measured in cell lysates by luminescence using the ATPlite assay kit (Perkin-Elmer), 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 0.1% DMSO. FCCP was used at 5 µM as a positive control. Two separate experiments were performed, each with three to four replicates per condition per time-point. Results were expressed as % ATP content of vehicle-treated wells.

Statistical Analysis

Data are reported as the mean ± s.e.m. of the indicated number of experiments. Results were analysed by one-way analysis of variance (ANOVA) using StatView software (SAS Institute Inc, Cary, NC, USA), with *post hoc* analysis as appropriate. Statistical significance was set at $p \leq 0.05$

Results

NSE Treatment Activates the Insulin Signalling Pathway in Muscle and Liver Cells

Insulin-dependent and -independent activation of Akt and ERK1/2, two major components of the insulin receptor signalling cascade [42], was assessed by western immunoblot with phospho-specific antibodies. C2C12 cells treated with NSE for 18 h showed increased contents of basal and insulin-stimulated (1 nM; 5 min) phospho-Akt and -ERK1/2, which persisted as far as 60 min after insulin stimulation (figure 1A). In fact, NSE treatment induced a proportional upward shift of content at every time-point (figure 1B). Contents of total Akt and ERK were not affected by the treatment (figure 1A).

In 3T3-L1 cells, NSE treatment had no effect on phospho-Akt or phospho-ERK contents (results not illustrated). No effect was seen on basal or insulin-stimulated states or on total adipocyte protein content in Akt or ERK1/2.

In H4IIE hepatocytes, NSE increased Akt phosphorylation by 1.5 fold (figure 2B), but not that of ERK1/2 (figure 2C). As in other tissues, NSE did not change the total content in unphosphorylated Akt and ERK1/2 in H4IIE cells (figure 2A, C).

NSE Treatment Activates the AMPK Signalling Pathway in Muscle and Liver Cells

NSE treatment increased the content of phosphorylated AMPKα (catalytic subunit of AMPK) and its downstream substrate ACC in C2C12 cells to levels comparable to that of AICAR (figure 3). Total AMPK was not altered. Similar results were observed following NSE treatment in H4IIE cells (figure 4). In 3T3-L1 adipocytes, however, NSE treatment did not increase phosphorylation of AMPK or ACC (data not shown).

Treatment with NSE Uncouples Oxidative Phosphorylation

As illustrated in figure 5 and quantified in table 1, NSE treatment was observed to uncouple oxidative phosphorylation of isolated liver mitochondria, as shown by an increase in basal (state 4) succinate-supported O_2 consumption immediately upon addition of extract. This effect was dose dependent and uncoupling was nearly complete at 200 µg/ml. The extract also exhibited a mild inhibitory activity, as shown by a slight decrease in ADP-stimulated (state 3) O_2 consumption (figure 5A; table 1). The activity of NSE was unaffected by pretreatment of mitochondria with the ANT inhibitor potassium atractyloside (AK), despite complete inhibition of state 3 respiration by AK (figure 5B). The activities of sequential solvent fractions as well as that of the oily and solid phases of NSE were also tested. The hexanolic and ethyl acetate fractions as well as the oily phase of the NSE exhibited conserved uncoupling activity (table 1). Known constituents

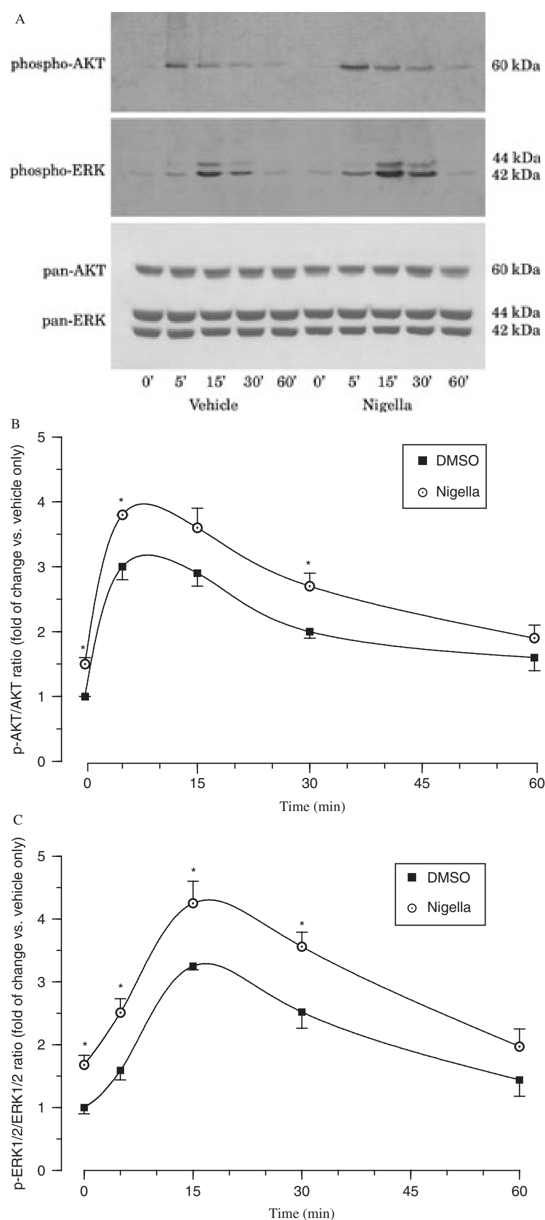

Figure 1. *Nigella sativa* seed extract (NSE) increases phosphorylation of Akt in C2C12 muscle cells. Differentiated C2C12 myotubes were treated with 200 µg/ml of NSE for 18 h. Cells were then treated for 5 min with 0 or 1 nM of insulin, followed by an additional incubation in Dulbecco's modified Eagle's medium (DMEM) for 5–55 min. A. Representative immunoblots. B. Quantitation of Akt immunoblots. C) Quantitation of ERK immunoblots. For B and C, data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± s.e.m. for n = 3 passages of cells. The symbol '*' indicates significantly different ($p < 0.05$) from vehicle control.

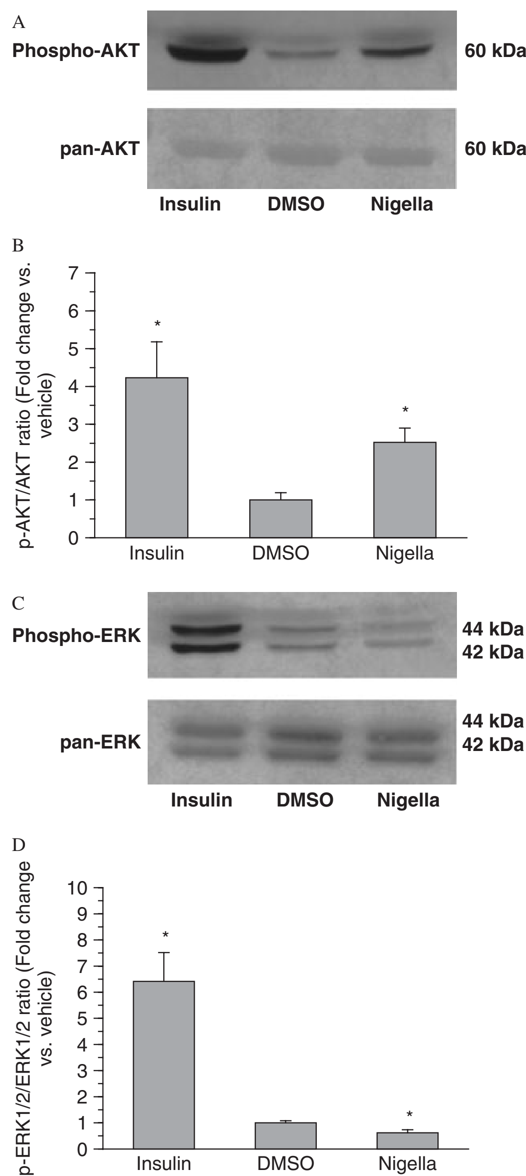

Figure 2. *Nigella sativa* seed extract (NSE) increases phosphorylation of Akt in H4IIE hepatocytes. H4IIE hepatocytes were treated with 200 µg/ml of NSE for 18 h. A 100 nM of insulin (100 nM; 15 min) served as a positive control. A. Representative Akt immunoblots. B. Quantitation of Akt immunoblots. C. Representative ERK immunoblots. D. Quantitation of ERK immunoblots. For B and D, data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± s.e.m. for n = 3 passages of cells. The symbol '*' indicates significantly different ($p < 0.05$) from vehicle control.

thymol, carvacrol, hederagenin, nigellimine and thymoquinone exhibited little or no uncoupling activity at 100 µM (table 1).

NSE Treatment Activates PPARγ Signalling Pathway

Using a luciferase gene reporter assay in HEK 293-T cells, NSE was observed to stimulate PPARγ activity in a logarithmic dose–response fashion, the effect reaching statistical significance at 50 and 200 µg/ml (figure 6).

Rate of Acidification of Extracellular Medium is not Increased by NSE

In H4IIE hepatocytes and C2C12 muscle cells, NSE treatment did not increase the rate of extracellular acidification as compared to vehicle control, whereas the positive control FCCP

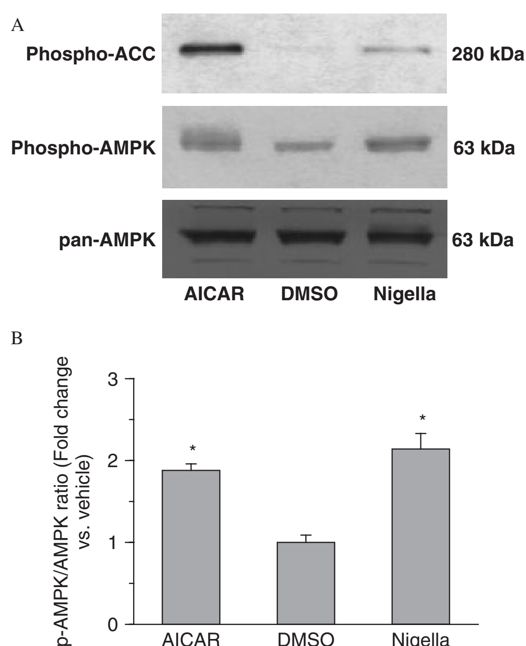

Figure 3. *Nigella sativa* seed extract (NSE) increases phosphorylation of AMP-activated protein kinase (AMPK) and acetyl CoA carboxylase (ACC) in C2C12 myotubes. C2C12 myotubes were treated 18 h with 200 μg/ml of NSE or 0.1% dimethyl sulfoxide (DMSO). A 2 mM of 5-aminoimidazole-4-carboxamide-1-b-D-rubofuranozide (AICAR) (2 mM; 30 min) served as a positive control. A. Representative immunoblots. B. Quantitation of results of p-AMPK immunoblots where data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± s.e.m. for n = 3 passages of cells. The symbol '*' indicates significantly different ($p < 0.05$) from vehicle control.

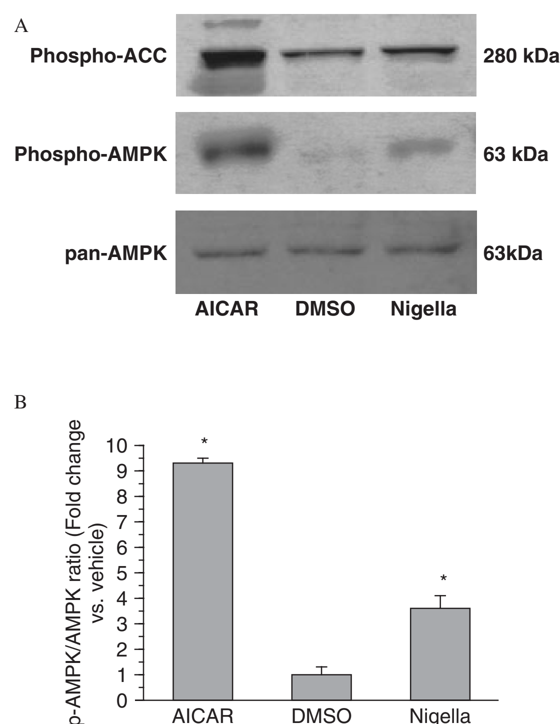

Figure 4. *Nigella sativa* seed extract (NSE) increases phosphorylation of AMP-activated protein kinase (AMPK) and acetyl CoA carboxylase (ACC) in H4IIE hepatocytes. H4IIE hepatocytes were treated 18 h with 200 μg/ml of NSE or 0.1% dimethyl sulfoxide (DMSO). A 2 mM of 5-aminoimidazole-4-carboxamide-1-b-D-rubofuranozide (AICAR) (2 mM; 30 min) served as a positive control. A. Representative immunoblots. B. Quantitation of results of p-AMPK immunoblots where data are integrated densities (arbitrary units) expressed as normalized ratios of phospho to total content. Data are mean ± s.e.m. for n = 3 passages of cells. The symbol '*' indicates significantly different ($p < 0.05$) from vehicle control.

caused a substantial increase in culture medium acidification rate (data not illustrated).

Cytosolic ATP Concentration is not Depressed by NSE

NSE treatment did not induce a decrease in ATP content in muscle cells (figure 7A). Rather, ATP content was slightly increased at 1 h and this increase was sustained up to 6 h. The reference uncoupler FCCP (5 μM) caused a decrease in ATP content of approximately 20% at 0.5 h, which was normalized by 1 h. Furthermore, like NSE, FCCP induced an overshoot effect observable at 3 h. In hepatocytes, effects were generally more important (figure 7B). FCCP caused a sustained drop in ATP content by approximately one third. NSE also caused a decrease in ATP but this was smaller and shorter-lived than that of FCCP.

Discussion

N. sativa seeds are used in the traditional medicine of numerous Middle Eastern and North African countries [29]. This action has been attributed to insulinotropic effects, as observed with NSE in isolated pancreatic islets [28] and in two pancreatic β-cell lines [30], as well as extrapancreatic effects, as observed *in vivo* [29] and in cultured skeletal muscle cells and adipocytes [30]. The present study focused on extrapancreatic effects and aimed to elucidate the molecular targets of NSE in C2C12 skeletal muscle cells, 3T3-L1 adipocytes and H4IIE hepatocytes.

We have previously demonstrated [30] that glucose uptake in the absence of insulin is greatly enhanced by an 18-h treatment with NSE in muscle cells and adipocytes; in both cell lines, this effect on basal uptake was equal to or greater than the effect of stimulation with 100 nM of insulin for 30 min. The goal of the present study was to identify the signalling pathway that could underlie these NSE-induced enhancements in glucose uptake. We first tested whether the insulin-like effect of NSE was mediated through the insulin signalling pathway. In skeletal muscle cells and adipocytes, insulin signals the translocation of Glut4 glucose transporters to the plasma membrane to increase glucose uptake [42]. In hepatocytes, insulin signals the inhibition of hepatic glucose production [43]. In the three target tissues, we assessed the degree of phosphorylation of Akt, a reliable marker of activity through the insulin signalling pathway. We also assessed the phosphorylation of ERK, a signalling protein involved in the anabolic effects of insulin. Our results show that in skeletal muscle and liver cells, but not in adipocytes, NSE increases basal phosphorylation of Akt in the absence of insulin. Moreover, this increase is additive to the

Table 1. Effects of ethanol extract, fractions and constituent compounds of *Nigella sativa* seeds on oxidative phosphorylation in isolated mitochondria.

N. sativa seed product	Uncoupling effect	Residual ATP synthetic capacity
Ethanol extract (NSE) (200 μg/ml)	64% ± 8	11% ± 6
NSE liquid phase only (140 μg/ml)	81% ± 5	0% ± 0
NSE solid phase only (60 μg/ml)	23% ± 2	42% ± 6
Hexane fraction (200 μg/ml)	64% ± 9	0% ± 0
Ethyl acetate fraction (200 μg/ml)	31% ± 5	0% ± 0
Methanol fraction (200 μg/ml)	2%	51%
Methanol/water fraction (200 μg/ml)	5%	75%
Carvacrol (100 μM)	7% ± 2	83% ± 3
Hederagenin (100 μM)	5%	83%
Nigellimine (100 μM)	3%	88%
Thymol (100 μM)	6% ± 0	66% ± 4
Thymoquinone (100 μM)	0%	90%

NSE, *Nigella sativa* seed extract.
Note: Respiration data are presented as mean ± s.e.m for two separate experiments performed in triplicate on different mitochondrial preparations. 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.

acute effect of insulin in skeletal muscle cells. Phosphorylation of ERK mirrored these results in skeletal muscle. However, ERK was not activated by NSE in hepatocytes or adipocytes.

We next tested whether activation of the AMPK pathway also contributed to the insulin-like effects of NSE in muscle and liver cells, and whether this pathway could explain the effects in adipocytes. The function of AMPK is to detect and transduce perturbations in cellular energy homeostasis, triggering cytoprotective responses to counter metabolic stress [44]. Some of these responses, including the stimulation of glucose uptake in skeletal muscle cells and adipocytes and the inhibition of hepatic glucose output [45, 46], are very similar to those of insulin, making AMPK a key therapeutic target for insulin resistance and diabetes [44]. Our findings indicate that the AMPK pathway (AMPK and ACC, one of its key effectors) is activated by NSE treatment in C2C12 and H4IIE. However, this pathway was not activated in 3T3-L1 adipocytes. This tissue specificity of AMPK activation by NSE is interesting and warrants further study, especially since AMPK activation in adipocytes has been implicated in the control of adiposity [47].

In light of the fact that the AMPK pathway is responsive to perturbations in energy homeostasis, we tested whether NSE could disrupt mitochondrial energy transduction and produce metabolic stress. Indeed, many natural products and naturally occurring compounds are known to inhibit or uncouple mitochondrial oxidative phosphorylation [48–50]. We treated isolated liver mitochondria with NSE and observed uncoupling activity. This effect was dose dependent and almost complete at the NSE concentration used to demonstrate activation of AMPK and an enhancement of glucose uptake in cells.

Nevertheless, targeting mitochondrial function is inherently dangerous as aerobic energy production capacity can easily fall below cellular energy demand. This can be compensated to some degree by anaerobic glycolysis [51]. However, the end product of this pathway is lactic acid which can potentially produce systemic acidosis [51]. We therefore tested the effects of NSE on the rate of acidification of the culture medium of muscle and liver cells. In both cell types, the rate of acidification was not different from vehicle and was substantially lower than that of the classic uncoupler FCCP tested at 5 μM. We also assessed the cytosolic concentration of ATP. One hour after onset of treatment, at a time when ATP is significantly depressed by FCCP, NSE was without effect. These findings suggest that the uncoupling activity of NSE may not be threatening and that treatment with *N. sativa* seeds is unlikely to cause systemic acidosis. One way to reconcile powerful uncoupling activity with only mild metabolic stress is to evoke short lasting actions that may be explained by rapid metabolism of active principles. Further studies will be necessary to determine if such considerations apply to NSE. Nevertheless, the fact that *N. sativa* can reduce metabolic efficiency without causing undue metabolic stress represents a highly desirable trait in the context of the metabolic syndrome and T2D.

While *N. sativa* seeds and seed oil are phytochemically well-characterized natural products [52], their active principle(s) have yet to be ascertained. To begin addressing this point, we used the crude fractionation of *N. sativa* seeds based on the classical approach of sequential extraction with solvents of increasing polarity. In agreement with most studies on glucose-lowering effects of *N. sativa* [29], the most lipophilic fractions demonstrated the largest uncoupling activity in isolated mitochondria and, correspondingly, the largest increase in glucose transport (data not shown).

We also considered that one of the common mechanisms of uncoupling involves the protonophoric activity of lipophilic weak acids [53] with acid dissociation constant (pKa1) ranging from 6.5 to 8.5 and moderate to high lipophilicity [water–octonol partition coefficient ($\log P \geq 2$)]; properties ideal for proton shuttling and resulting metabolic inefficiency. However, no heretofore reported constituent of *N. sativa* seeds can be predicted to exhibit the desired physicochemical properties. For instance, hederagenin ($\log P = 5.3$; $pKa1 = 4.7$) is a carboxyl containing triterpene, whose low pKa1 is incompatible with protonophoric activity, and we consequently found the compound to have no uncoupling activity. Similarly, we tested some small phenolic compounds that have been identified in *N. sativa*, and even proposed to be bioactive [54–56], but that would not be good protonophores given the absence of ionizable groups or clearly incompatible pKa1. This included thymoquinone, carvacrol ($\log P = 3.4$; $pKa1 = 10.4$), thymol ($\log P = 3.4$; $pKa1 = 10.6$) and nigellimine ($\log P = 1.6$; $pKa1 = 7.0$), none of which exerted a significant uncoupling activity.

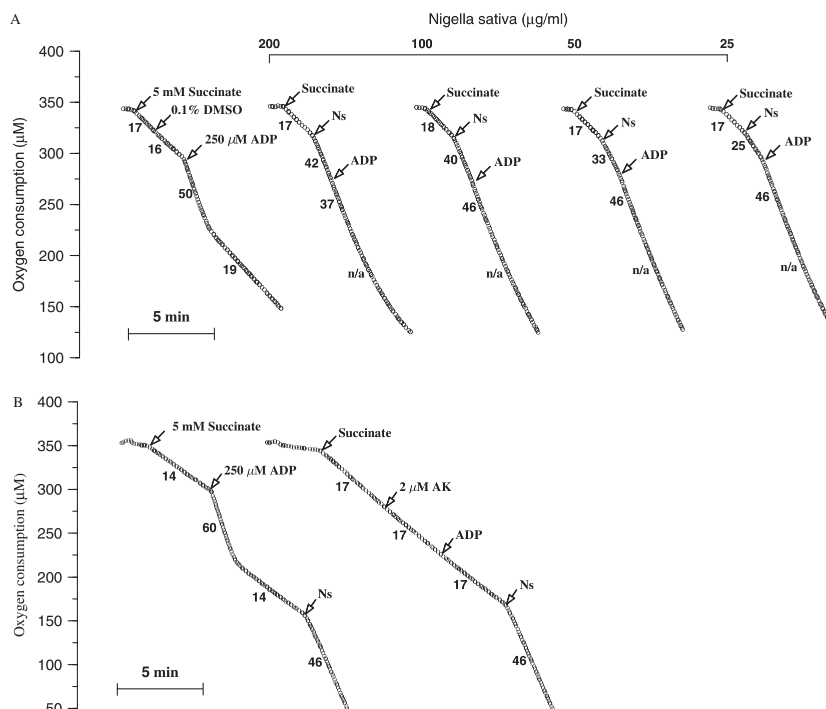

Figure 5. *Nigella sativa* seed extract (NSE) uncouples oxidative phosphorylation in isolated rat liver mitochondria. A) Representative tracings of the effects of vehicle [0.1% dimethyl sulfoxide (DMSO)] or NSE (25–200 μg/ml). NSE significantly increases the rate of basal oxygen consumption (i.e. uncoupling effect) in a dose-dependent manner, while reducing the rate of ADP-stimulated oxygen consumption (i.e. inhibitory effect). At 200 μg/ml, effects combine and completely abolish the ATP synthetic capacity of NSE-treated mitochondria. B) Representative tracings of the effects of vehicle or NSE in the presence of atractyloside potassium salt (AK), an inhibitor of the adenine nucleotide translocase (ANT). Pretreatment with AK does not block the uncoupling effect of NSE.

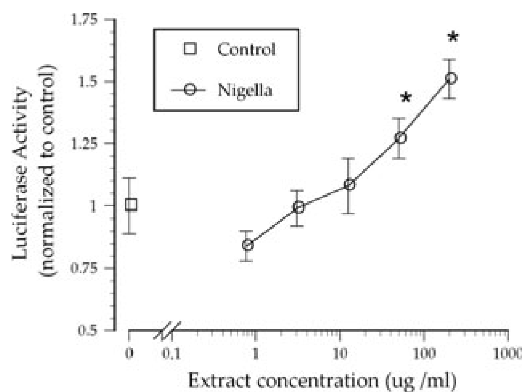

Figure 6. *Nigella sativa* seed extract (NSE) stimulates peroxisome proliferator-activated receptor (PPAR) activity. NSE increased PPARγ-dependent luciferase activity in a dose-dependent manner by up to 50% at 200 μg/ml. This effect, while significant, is much smaller than that of the PPARγ agonist, rosiglitazone, used as a positive control (800 nM; not illustrated). The symbol '*' indicates significantly different ($p < 0.05$) from vehicle control.

This suggests that the powerful uncoupling activity of NSE may (i) result from the additive effect of several mild uncouplers; (ii) may be attributable to compounds other than the ones tested or (iii) may be attributable to another mechanism such as the interaction with a transmembrane protein of the inner mitochondrial membrane [57], notably the ANT. Interaction with the ANT was directly ruled out since the ANT inhibitor atractyloside (2 μM) had no effect on the activity of NSE. Further studies will be necessary to clarify the components of *N. sativa* that are responsible for its uncoupling effect.

Nonetheless, the compound thymoquinone, suggested by several groups to be an active principle of *N. sativa* [58], was found to stimulate glucose uptake in muscle cells up to a maximum of 29% at a dose of 30 μM (less than crude NSE; data not shown), despite its lack of effect on basal mitochondrial respiration, concordant with its lack of ionizable groups. One interpretation to reconcile these results is that thymoquinone could activate the insulin signalling pathway rather that implicating AMPK as does NSE. Further studies will be necessary to clarify this point.

Finally, using a reporter gene assay, we observed that NSE behaves like an agonist of PPARγ, increasing its activity by more than 50% at 200 μg/ml. While this effect was relatively small compared to that of rosiglitazone, it may be sufficient [59] to account, at least in part, for the stimulation of adipogenesis in adipocytes previously observed with NSE [30]. The present results also help reconcile the previous observation that in adipocytes, but not in skeletal muscle, NSE potentiated the effect of insulin on glucose transport [30].

In summary, NSE exerts insulin-like actions on skeletal muscle cells, hepatocytes and adipocytes through three major

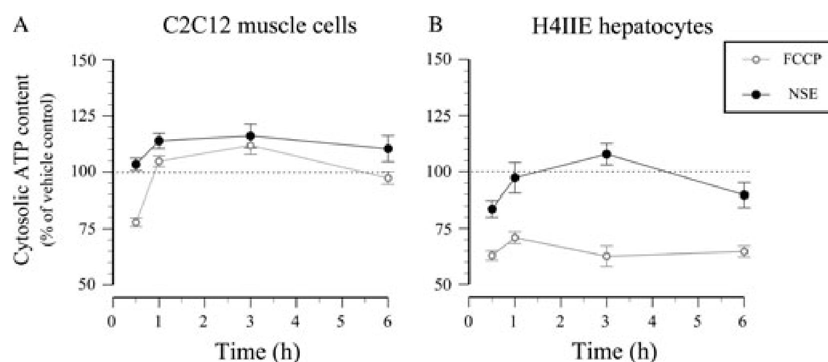

Figure 7. Cytosolic ATP concentration is normal or supranormal 1 h after onset of treatment with *Nigella sativa* seed extract (NSE). Cytosolic ATP content was measured in H4IIE hepatocytes and C2C12 muscle cells using a luminescent ATP assay. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (5 μM) was used as a positive control. Data are mean ± s.e.m. of two experiments of four to five replicates per condition per time-point.

pathways involved in glucose homeostasis: the insulin signalling pathway, the AMPK pathway and the PPARγ pathway. The trigger for the AMPK pathway appears to be the metabolic stress that results from the uncoupling of oxidative phosphorylation by NSE. However, the stress appears to be mild and short-lived suggesting that *N. sativa* seeds have low potential for inducing dangerous systemic acidosis. The identification of these effector pathways of the biological activity of NSE explains in large part the well-documented antihyperglycaemic effects of *N. sativa* and the use of this plant in the traditional medicine of several cultures. The results presented here support the notion that *N. sativa* has exceptional potential as a complementary or alternative approach to currently available antidiabetic medications.

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RESEARCH ARTICLE

Stimulation of AMP-activated protein kinase 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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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, 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.

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Abbreviations: ACC, acetyl coA carboxylase; **AICAR,** aminoimi dazole carboxamide ribonucleotide; **AMPK,** AMP activated protein kinase; **APCI,** atmospheric pressure chemical ionization; **FC,** func tional capacity; **FCCP,** carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone; **KPB,** Krebs phosphate buffer; **RASOC,** rate of ADP stimulated O2 consumption; **RBOC,** the rate of basal oxygen consumption

*These authors contributed equally to this work.

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1 Introduction

Aboriginal populations the world over 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 seden tary 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 it is these complications more than diabetes itself that 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 pharmacopeia 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 flavo noid [22] transiently inhibit mitochondrial ATPsynthase, leading to the activation of AMPK, and propose that quer cetin and quercetin glycosides are responsible for the anti diabetic activity of *V. vitis* and perhaps of other species of this genus.

2 Materials and methods

2.1 Plant material and extraction

Berries of *V. vitis idaea* L. (*V. vitis)* were collected in Whap magoostui, 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 etha nol 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.

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2.2 Cell culture

C2C12 murine skeletal myoblasts and H4IIE murine hepato cytes were obtained from the American Type Cell Collection (Manassas, VA, USA). Cell culture media were purchased from Invitrogen Life Technologies (Burlington, Ont., Canada) unless otherwise noted. Other reagents were purchased from Sigma Aldrich (Oakville, Ont., Canada) unless otherwise noted. C2C12 myoblasts were cultured in 6 or 12 well plates in high glucose DMEM supplemented with 10% fetal bovine serum, 10% horse serum and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) at 37°C in a 5% CO_2 atmo sphere. After 80% confluence, myoblasts were differentiated into myotubes in DMEM supplemented with 2% horse serum and antibiotics for exactly 7 days, resulting in the fusion of all cells into multinucleated myotubes. H4IIE hepatocytes were grown in 6 well plates in DMEM supplemented with 10% fetal bovine serum until fully confluent and experiments were performed 1 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.

2.3 Glucose uptake assay

The effects of plant products on the rate of uptake of glucose by differentiated C2C12 skeletal myotubes were assessed with a ^3H 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 phos phate 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 μCi/mL 2 deoxy D [1 ^3H] 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 radio activity was measured in a liquid scintillation counter (LKB Wallac 1219; Perkin Elmer, Woodbridge, Ont., Canada).

2.4 Western immunoblot

The effects of plant products on the insulin and AMPK signaling pathways of C2C12 muscle cells or H4IIE hepato cytes 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 orthovana date, 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% β mercaptoethanol, and 0.01% bromophenol blue). Briefly, 100 μL of each sample were separated on 10% polyacrylamide full size gels and transferred to poly vinylidene 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 second ary 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 simul taneously to a single membrane. Quantification of the inte grated 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).

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2.5 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 lapar otomized. 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 centri fuged at $600 \times g$ for 10 min at 4°C in order to remove cellular fragments and the resulting supernatant was centrifuged at $12\,000 \times g$ 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 consump tion *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 phos phorylation (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 mito chondrial 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 synth esis). 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 experi ment was expressed as a percentage of the average control FC for the session to give the percentage residual FC.

2.6 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: $pH = 0.765 \times \ln(A\ 530/A\ 450) + 7.61$ ($R^2 = 0.99$). 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. Experi ments 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

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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.

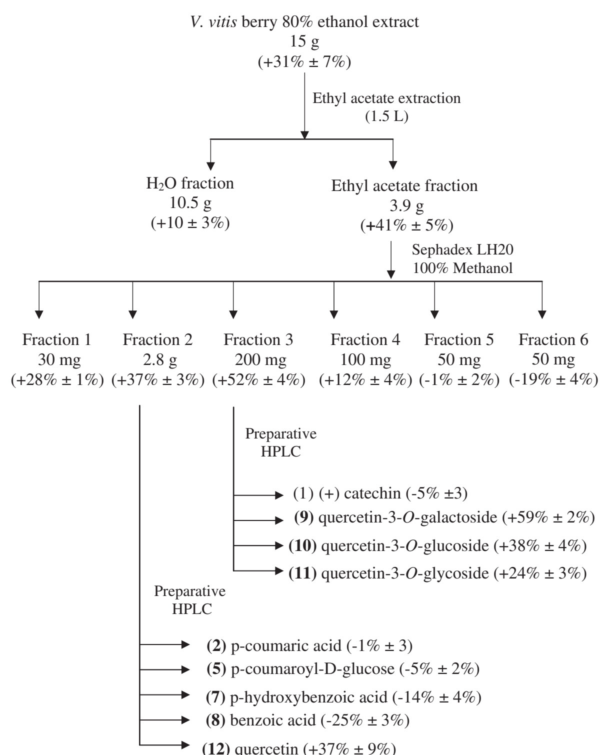

Figure 1. Phytochemical fractionation of *V. vitis* berry extract guided by muscle cell glucose uptake activity. Values in brackets represent activity expressed as percentage change in the rate of basal glucose uptake relative to the vehicle control (0.1% DMSO) following an 18 h treatment with respective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

2.7 Cytosolic ATP assay

Total cytosolic ATP was measured in cell lysates by lumi nescence 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 hepa tocytes 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.

2.8 Statistical analysis

Results are reported as means ± SEM, with the number of replicates and number of independent experiments indi cated. 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, USA). Statis tical significance was set at $p \leq 0.05$.

2.9 Fractionation, isolation and identification

Fractionation of the ethyl acetate soluble fraction of *V. vitis* berry ethanol extract is shown in Fig. 1. Gel filtration chro matography 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). Frac tions 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.

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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.

3 Results

3.1 *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 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).

3.2 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, eight 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).

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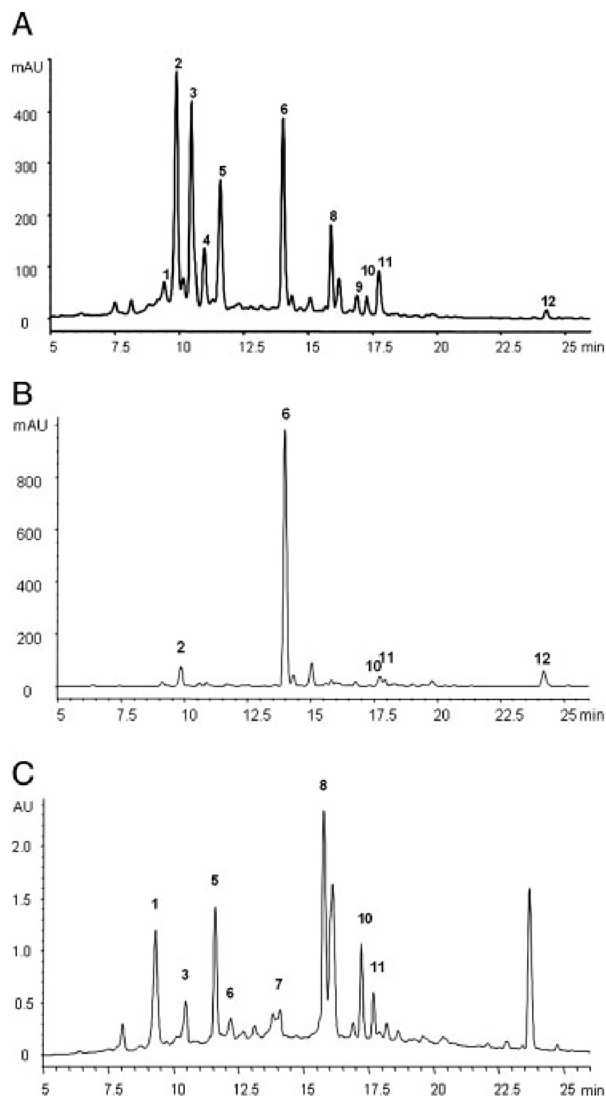

Figure 2. HPLC chromatograms of *V. vitis* berry crude extract (A), subfractions 2 (B) and 3 (C) of its ethyl acetate soluble fraction. Absorbance at 325 and 520 nm (milliabsorbance units) is plotted against retention time (in min). Twelve constituent compounds were identified using a metabolomics approach, as described in Section 2.1. The identity of these peaks is listed in Table 1.

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 glucoside	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	Unidentified quercetin 3 *O* glycoside	21.9±1.3
12	Quercetin	2.3±0.3

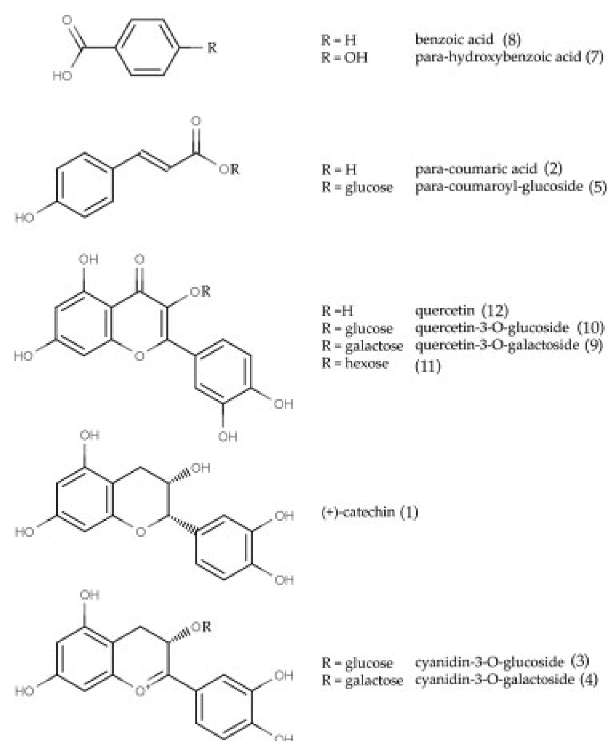

Figure 3. Chemical structures of the 12 isolated constituents *V. vitis* berry ethanol extract.

3.3 *V. vitis* crude extract and its actives 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 eval uated 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 activation 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* galacto side or quercetin 3 *O* glucoside did not increase phosphor ylation of Akt (Fig. 4A) but increased phosphorylation of ACC (Fig. 4B). Total content of ACC was not significantly altered by any treatment.

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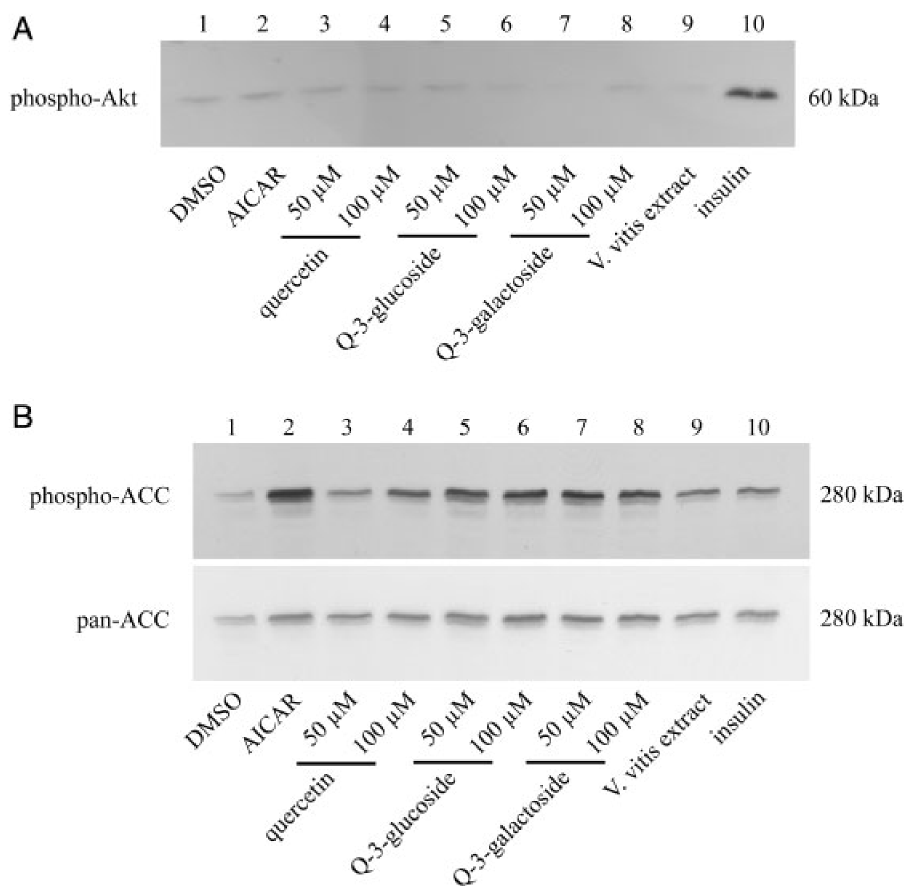

Figure 4. *V. vitis* berry extract and its active principles stimulate the AMPK signaling pathway but not the insulin receptor pathway. C2C12 skeletal muscle cells were treated for 18 h with either 0.1% DMSO (vehicle), 200 µg/mL of *V. vitis* berry extract, or 50 and 100 µM of quercetin, quercetin 3 *O* glucoside or quercetin 3 *O* galacto side. 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.

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

AMPK is highly sensitive to metabolic stress such as can occur 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 ADP stimulated O_2 consump tion (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

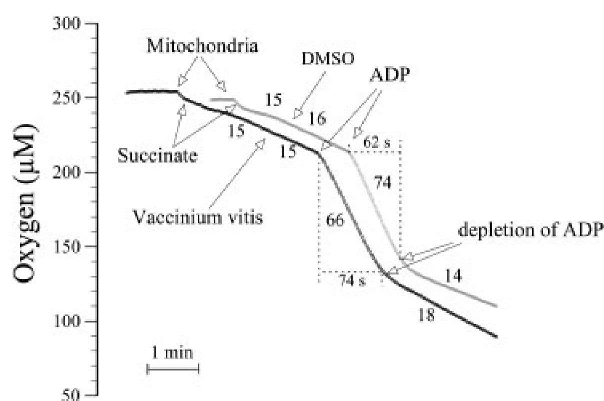

Figure 5. The *V. vitis* berry extract induces a mild instantaneous inhibition of respiration in isolated rat liver mitochondria, as illu strated by a representative oxygen consumption tracing. Mitochon dria (1 mg mitochondrial protein) were treated with vehicle (0.1% DMSO) or 200 µg/mL of extract and 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.

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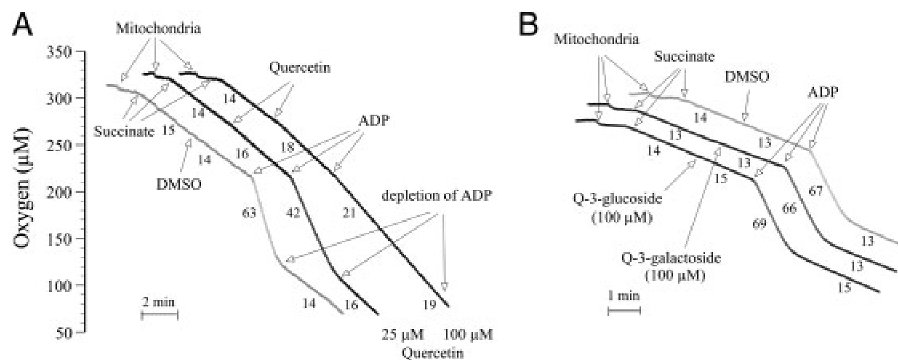

Figure 6. Quercetin (A), but not quercetin 3 *O* glycosides (B), induces an important instantaneous and dose dependent inhibition of respiration in rat liver mitochondria, as illustrated by representative oxygen consumption tracings. Values represent rate of consumption in nmol O/min/mg protein. Experiments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

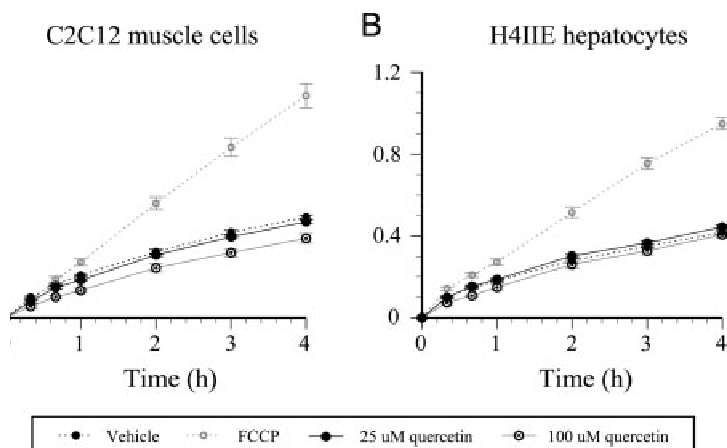

Figure 7. Quercetin does not increase the rate of secretion of acid equivalents by C2C12 (A) or H4IIE (B) cells. Acidification of the cell medium. The pH of the culture medium was assessed with a Phenol Red based spectrophotometric assay at several time points. Change in pH was expressed as the cumulative 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.

aglycone, only decreasing capacity by 3 7% at 100 µM (Fig. 6B).

3.5 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.

4 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 pharmacopeia. 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].

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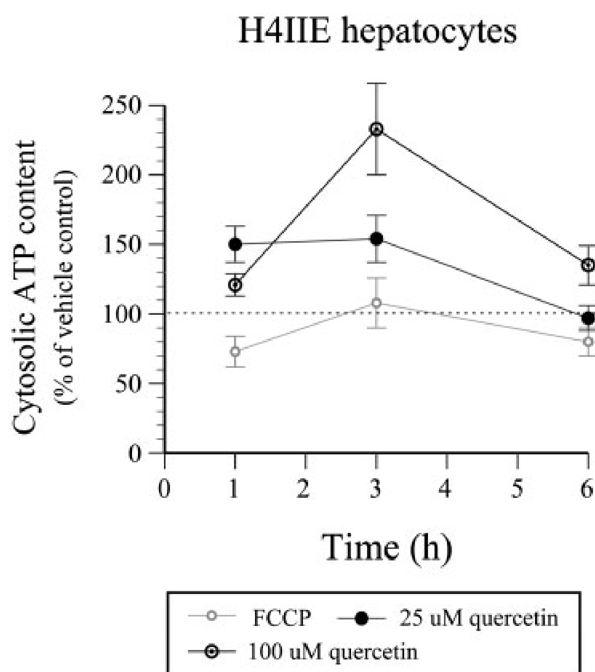

Figure 8. Quercetin does not reduce intracellular ATP concentration in H4IIE hepatocytes. Cytosolic ATP content was measured in H4IIE hepatocytes using a luminescent ATP assay. FCCP (5 μM) was used a positive control. Quercetin treatment was not significantly different from vehicle (0.1% DMSO) at either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

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. Some more 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 others have been shown to inhibit electron transport or ATPsynthase [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

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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 quer cetin 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 concen tration 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 meta bolized compound, a conclusion supported by pharmacoki netic studies [22].

5 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 abori ginal populations.

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